

5 NaCT AS A TARGET FOR LIFESPAN EXPANSION AND WEIGHT
REDUCTION

CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application
10 Serial No. 60/428,469, filed November 22, 2002, and U.S. Provisional
Application Serial No. 60/459,441, filed April 1, 2003, each of which is
incorporated by reference herein.

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20 BACKGROUND

Na⁺-coupled dicarboxylate transporters (NaDCs) mediate the cellular
entry of a variety of citric acid cycle intermediates in mammalian tissues. There
are two different isoforms of NaDC in mammals, namely NaDC1 and NaDC3
(Pajor, *J. Membrane Biol.* (2000);175: 1-8). While both are sodium-coupled
25 transporters for succinate and other dicarboxylate intermediates of citric acid
cycle (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682), these transporters can be
distinguished from one another primarily based on their substrate affinity.
NaDC1 is a low affinity transporter with a Michaelis-Menten constant (K_m) for
succinate in the millimolar range, whereas NaDC3 is a high affinity transporter
30 with a K_m for succinate in the micromolar range. NaDC2, identified in *Xenopus*
laevis intestine, is functionally and structurally related to the mammalian
NaDCs, but may represent a species-specific ortholog of NaDC1 (Bai and Pajor,
Am. J. Physiol. (1997);273: G267-G274).

The mammalian NaDCs have been cloned from different species and their functional characteristics have been elucidated in different heterologous expression systems. See, for example, Pajor, *J. Biol. Chem.* (1995);270: 5779-5785; Pajor, *Am. J. Physiol.* (1996);270: F642-F648; Chen et al., *J. Biol. Chem.* (1998);273: 20972-20981; Kekuda et al., *J. Biol. Chem.* (1999);274: 3422-3429; Wang et al., *Am. J. Physiol.* (2001);278: C1019-C1030; Chen et al., *J. Clin. Invest.* (1999);103: 1159-1168; Pajor et al., *Am. J. Physiol.* (2001);280: C1215-C1223; and Pajor and Sun, *Am. J. Physiol.* (2000);279: F482-F490. The Na⁺-coupled dicarboxylate transporters ceNaDC1 and ceNaDC2 from the nematode *C. elegans* have also been cloned. See, Fei et al., (2003) *J Biol Chem* 278, 6136-6144.

Recently, Rogina et al. (Rogina et al., *Science* (2000);290: 2137-2140) reported on the identification of a gene in *Drosophila melanogaster* which, when mutated, confers life span extension to the organism. Interestingly, the predicted protein product of this gene, known as Indy (for *I'm Not Dead Yet*), shows significant homology to mammalian NaDCs. It was therefore suggested that Indy is the *Drosophila* ortholog of either NaDC1 or NaDC3. However, even though it was assumed, based on the structural similarity, that *Drosophila* Indy is a sodium-coupled transporter for dicarboxylates similar to mammalian NaDCs, its transport identity has not been established.

NaDC1 is expressed primarily in the intestine and kidney, whereas NaDC3 is expressed, not only in the intestine and kidney, but also in the brain, liver, and placenta (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682). A unique feature of both of these transporters is that they interact with dicarboxylates with greater preference than with citrate, a tricarboxylate at physiological pH. Furthermore, even though NaDC1 and NaDC3 are able to transport citrate to some extent, only the dianionic form of citrate is recognized as the substrate by these transporters. Thus, NaDC1 and NaDC3 are specific for dicarboxylates. NaDCs are structurally related to the Na⁺-coupled sulfate transporters NaSi and SUT1 (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682). Together, these transporters constitute the NaDC/NaSi gene family.

While NaDC1, as well as NaDC3, can transport citrate to some extent, the efficiency of transport is low because these transporters recognize only the

dicarboxylate form of citrate as substrate. Since the dicarboxylates, such as succinate, malate, fumarate, oxaloacetate, and α -ketoglutarate, are present in blood at low levels, the high-affinity transporter NaDC3 is ideally suited for efficient transport of these substrates under physiological conditions. However, the circulating levels of citrate are several-fold greater than the combined levels of the dicarboxylates. The concentration of citrate in blood is approximately 135 μ M. In contrast, the concentration of succinate is approximately 40 μ M and the concentrations of other dicarboxylates are even lower. The divalent form of citrate, which is recognized by NaDC3 as a substrate, is present only at low concentrations (approximately 10 μ M) in blood at physiological pH. Therefore NaDC3 does not provide an efficient mechanism for the cellular utilization of citrate present in the circulation.

SUMMARY OF THE INVENTION

The present invention includes the identification and characterization of a novel transmembrane transporter, a Na^+ -coupled citrate transporter ("NaCT"), which recognizes the tricarboxylate citrate with higher affinity than the dicarboxylates, such as succinate, malate, fumarate, and 2-oxo-glutarate. The present invention also demonstrates, for the first time, that the Na^+ -coupled citrate transporter is a target for lithium action, with the action of lithium on NaCT varying depending on the animal species. Human NaCT is activated by lithium, while rodent NaCTs are inhibited by lithium.

The present invention includes isolated polynucleotides encoding a polypeptide having at least 35% sequence identity to SEQ ID NO:8, wherein the polynucleotide encodes a polypeptide demonstrating Na^+ -dependent transmembrane transport of citrate. In some embodiments, the polynucleotide includes SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

The present invention also includes isolated polynucleotides that hybridize to SEQ ID NO:1 under stringent hybridization conditions, wherein the polynucleotide encodes a polypeptide demonstrating transmembrane transport

of citrate. In some embodiments, the polynucleotide includes SEQ ID NO:1. In some embodiments, the polynucleotide does not include SEQ ID NO:1.

The present invention includes isolated polynucleotides that hybridize to SEQ ID NO:3 under stringent hybridization conditions, wherein the
5 polynucleotide encodes a polypeptide demonstrating Na⁺-dependent transmembrane transport of citrate. In some embodiments, the polynucleotide includes SEQ ID NO:3.

The present invention includes isolated polynucleotides that hybridize to SEQ ID NO:5 under stringent hybridization conditions, wherein the
10 polynucleotide encodes a polypeptide demonstrating Na⁺-dependent transmembrane transport of citrate. In some embodiments the isolated polynucleotide includes SEQ ID NO:5.

The present invention also includes isolated polynucleotides that hybridize to SEQ ID NO:7 under stringent hybridization conditions, wherein the
15 polynucleotide encodes a polypeptide demonstrating Na⁺-dependent transmembrane transport of citrate. In some embodiments, the isolated polynucleotide includes SEQ ID NO:7.

The present invention also includes isolated polynucleotides that hybridize to SEQ ID NO:9 under stringent hybridization conditions, wherein the
20 polynucleotide encodes a polypeptide demonstrating Na⁺-dependent transmembrane transport of citrate. In some embodiments, the isolated polynucleotide includes SEQ ID NO:9.

The present invention also includes isolated polynucleotides that hybridize to SEQ ID NO:11 under stringent hybridization conditions, wherein
25 the polynucleotide encodes a polypeptide demonstrating Na⁺-dependent transmembrane transport of citrate. In some embodiments, the isolated polynucleotide includes SEQ ID NO:11.

Also included in the present invention are isolated polynucleotides encoding a polypeptide having at least 35% sequence identity to SEQ ID NO:6,
30 wherein the polynucleotide encodes a polypeptide demonstrating Na⁺-dependent transmembrane transport of citrate. In some embodiments, the isolated polynucleotide includes SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11. In some embodiments, the Na⁺-dependent

transmembrane transport of citrate is modulated by Li^+ . In some embodiments, the Na^+ -dependent transmembrane transport of citrate demonstrates a requirement for multiple Na^+ ions for transport coupling. In some embodiments, the transmembrane transport of citrate is electrogenic.

5 Also included in the present invention are plasmids with a polynucleotide encoding a polypeptide demonstrating transmembrane transport of citrate. In some embodiments, the plasmid includes an expression vector.

The present invention also includes host cells including a polynucleotide encoding a Na^+ -dependent transmembrane citrate transporter. In some
10 embodiments, the host cell demonstrates transient expression of the encoded Na^+ -dependent transmembrane citrate transporter. In some embodiments, the host cell demonstrates stable expression of the encoded Na^+ -dependent transmembrane citrate transporter. In some embodiments, the encoded Na^+ -dependent transmembrane transport of citrate is modulated by Li^+ . In some
15 embodiments, the isolated host cell may be a human cell, an insect cell, a *Xenopus* oocyte, or a yeast cell.

The present invention also includes isolated polypeptides having at least 35% identity with SEQ ID NO:2, wherein the polypeptide is a transmembrane transporter of citrate. In various embodiments, the isolated polypeptide includes
20 SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12. In some embodiments, the polypeptide demonstrates Na^+ -dependent transmembrane transport of citrate. In some embodiments, the Na^+ -dependent transmembrane transport of citrate is modulated by Li^+ .

The present invention also includes isolated polypeptides, wherein the
25 polypeptide is encoded by a polynucleotide that hybridizes to SEQ ID NO:1 under stringent hybridization conditions and wherein the polypeptide demonstrates transmembrane transport of citrate.

The present invention includes isolated polypeptides having at least 35% sequence identity to SEQ ID NO:6, wherein the polypeptide demonstrates Na^+ -
30 dependent transmembrane transport of citrate. In some embodiments, the Na^+ -dependent transmembrane transport of citrate is modulated by Li^+ . In some embodiments, the Na^+ -dependent transmembrane transport of citrate

demonstrates a requirement for multiple Na⁺ ions for transport coupling. In some embodiments, the transmembrane transport of citrate is electrogenic.

The present invention also includes isolated polypeptides having at least 35% sequence identity to SEQ ID NO:8, wherein the polypeptide demonstrates Na⁺-dependent transmembrane transport of citrate.

Also included in the present invention are antibodies that specifically bind to a polypeptide that demonstrates Na⁺-dependent transmembrane transport of citrate. In some embodiments, the antibody is monoclonal or polyclonal. In some embodiments, the antibody is derived from a mouse, rat, rabbit, hamster, goat, horse, or human. In some embodiments, the antibody is produced recombinantly. In some embodiments, one or more variable regions from the antibody are included in a chimeric protein. In some embodiments, the antibody is linked to a detectable marker.

The present invention also includes a method of identifying an agent that modifies transmembrane citrate transporter activity, the method including contacting a host cell expressing a transmembrane citrate transporter polypeptide having at least 35% identity with SEQ ID NO:2 with an agent; measuring citrate transport into the host cell in the presence of agent; and comparing citrate transport into the host cell in the presence of the agent to citrate transport into the host cell in the absence of the agent; wherein a decreased transport of citrate into the host cell in the presence of the agent indicates the agent is an inhibitor of transmembrane citrate transporter activity; wherein an increased transport of citrate into the host cell in the presence of the agent indicates the agent is a stimulator of transmembrane citrate transporter activity.

The present invention also includes a method of identifying an agent that modifies transmembrane citrate transporter activity, the method including contacting a host cell expressing a transmembrane citrate transporter polypeptide having at least 35% sequence identity to SEQ ID NO:8, wherein the transmembrane citrate transporter polypeptide demonstrates Na⁺-dependent transmembrane transport of citrate; measuring citrate transport into the host cell in the presence of agent; and comparing citrate transport into the host cell in the presence of the agent to citrate transport into the host cell in the absence of the

agent; wherein a decreased transport of citrate into the host cell in the presence of the agent indicates the agent is an inhibitor of transmembrane citrate transporter activity; wherein an increased transport of citrate into the host cell in the presence of the agent indicates the agent is a stimulator of transmembrane
5 citrate transporter activity.

The present invention includes a method of identifying an agent that modifies transmembrane citrate transporter activity, the method including contacting a host cell expressing a transmembrane citrate transporter polypeptide having at least 35% sequence identity to SEQ ID NO:6, wherein the
10 transmembrane citrate transporter polypeptide demonstrates Na⁺-dependent transmembrane transport of citrate and wherein the encoded Na⁺-dependent transmembrane transport of citrate is stimulated by Li⁺; measuring citrate transport into the host cell in the presence of agent; and comparing citrate transport into the host cell in the presence of the agent to citrate transport into
15 the host cell in the absence of the agent; wherein a decreased transport of citrate into the host cell in the presence of the agent indicates the agent is an inhibitor of transmembrane citrate transporter activity; wherein an increased transport of citrate into the host cell in the presence of the agent indicates the agent is a stimulator of transmembrane citrate transporter activity. In some embodiments, the transmembrane citrate transporter polypeptide includes SEQ ID NO:6. In
20 some embodiments, the present invention includes a modifier of a transmembrane citrate transporter, as identified by the method.

The present invention also includes a modifier of a transmembrane citrate transporter. In some embodiments, the transmembrane citrate transporter
25 has SEQ ID NO:6. In some embodiments, the modifiers may be included in a composition, including compositions that include a pharmaceutically acceptable carrier. In some embodiments, the composition may include an additional therapeutic agent, including, for example, lithium.

The present invention includes a method of extending the lifespan in a
30 subject by administering an inhibitor of a transmembrane citrate transporter to a subject.

The present invention includes a method of weight reduction in a subject by administering an inhibitor of a transmembrane citrate transporter to a subject.

The present invention includes a method of preventing weight gain in a subject by administering an inhibitor of a transmembrane citrate transporter to a subject. In some embodiments, the subject may be a human subject or a domestic pet.

5 The present invention includes a method of lowering blood cholesterol levels in a subject by administering an inhibitor of a transmembrane citrate transporter to a subject.

 The present invention includes a method of lowering blood triglyceride levels in a subject by administering an inhibitor of a transmembrane citrate
10 transporter to a subject.

 The present invention includes a method of lowering blood LDL levels in a subject by administering an inhibitor of a transmembrane citrate transporter to a subject.

 The present invention includes a method of lowering blood glucose
15 levels in a subject by administering an inhibitor of a transmembrane citrate transporter to a subject. In some embodiments, the subject is a diabetic.

 The present invention includes a method of identifying an agent that modifies Na^+ -dependent transmembrane citrate transporter activity, the method including contacting a host cell expressing a Na^+ -dependent transmembrane
20 citrate transporter selected from the group consisting of SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, and SEQ ID NO:12 with an agent; measuring the citrate-induced inward electrical current into the host cell in the presence of agent; and comparing the citrate-induced inward electrical current into the host cell in the presence of the agent to the citrate-induced inward
25 electrical current into the host cell in the absence of the agent; wherein a decrease in the inward electrical current into the host cell in the presence of the agent indicates the agent is a blocker of Na^+ -dependent transmembrane citrate transporter activity; wherein an increase in the inward electrical current into the host cell in the presence of the agent indicates the agent is a stimulator of Na^+ -
30 dependent transmembrane citrate transporter activity.

 The present invention includes a method of identifying an agent that serves as a substrate of a Na^+ -dependent transmembrane citrate transporter, the

method including contacting a host cell expressing a Na⁺-dependent transmembrane citrate transporter selected from the group consisting of SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, and SEQ ID NO:12 with an agent; and determining the entry of the agent into the cell via the Na⁺-
5 dependent transmembrane citrate transporter in the presence of agent; wherein entry of the agent via the Na⁺-dependent transmembrane citrate transporter indicates the agent is a substrate of a Na⁺-dependent transmembrane citrate transporter.

10 Definitions

As used herein, the term "isolated" means that a polynucleotide or polypeptide is either removed from its natural environment or synthetically derived, for instance by recombinant techniques, or chemically or enzymatically synthesized. An isolated polynucleotide denotes a polynucleotide that has been
15 removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Isolated polynucleotides of the present invention are free of other coding sequences with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions
20 such as promoters and terminators. Preferably, the polynucleotide or polypeptide is purified, i.e., essentially free from any other polynucleotides or polypeptides and associated cellular products or other impurities.

"Polynucleotide" and "nucleic acid sequences" are used interchangeably to refer to a linear polymeric form of nucleotides of any length, either
25 ribonucleotides or deoxynucleotides, and includes both double- and single-stranded DNA and RNA. A polynucleotide can be linear or circular in topology. A polynucleotide can be obtained using any method, including, without limitations, common molecular cloning and chemical nucleic acid synthesis. A polynucleotide may include nucleotide sequences having different
30 functions, including for instance coding sequences, and non-coding sequences.

As used herein "coding sequence," "coding region," and "open reading frame" are used interchangeably and refer to a polynucleotide that encodes a polypeptide, usually via mRNA, when placed under the control of appropriate

regulatory sequences. The boundaries of the coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end.

As used herein, "stringent hybridization conditions" refer to hybridization conditions such as 6X SSC, 5X Denhardt, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/ml fragmented and denatured salmon sperm DNA hybridized overnight at 65°C and washed in 2X SSC, 0.1% SDS at least one time at room temperature for about 10 minutes followed by at least one wash at 65°C for about 15 minutes followed by at least one wash in 0.2X SSC, 0.1% SDS at room temperature for at least 3-5 minutes. Typically, a 20X SSC stock solution contains about 3M sodium chloride and about 0.3M sodium citrate.

As used herein, "complement" and "complementary" refer to the ability of two single stranded polynucleotides to base pair with each other, where an adenine on one polynucleotide will base pair to a thymine on a second polynucleotide and a cytosine on one polynucleotide will base pair to a guanine on a second polynucleotide. Two polynucleotides are complementary to each other when a nucleotide sequence in polynucleotide can base pair with a nucleotide sequence in a second polynucleotide. For instance, 5'-ATGC and 5'-GCAT are complementary. Typically two polynucleotides are complementary if they hybridize under the standard conditions referred to herein.

"Polypeptide," as used herein, refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide, whether naturally occurring or synthetically derived, for instance, by recombinant techniques or chemically or enzymatically synthesized. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like. The following abbreviations are used throughout the application:

A = Ala = Alanine	T = Thr = Threonine
V = Val = Valine	C = Cys = Cysteine
L = Leu = Leucine	Y = Tyr = Tyrosine
I = Ile = Isoleucine	N = Asn = Asparagine

P = Pro = Proline	Q = Gln = Glutamine
F = Phe = Phenylalanine	D = Asp = Aspartic Acid
W = Trp = Tryptophan	E = Glu = Glutamic Acid
M = Met = Methionine	K = Lys = Lysine
5 G = Gly = Glycine	R = Arg = Arginine
S = Ser = Serine	H = His = Histidine

A “subject” or an “individual” is an organism, including, for example, a microbe, a plant, an invertebrate, or a vertebrate, such as, but not limited to, an animal. An animal may include, for example, a bird, a fish, a rat, a mouse, a domestic pet, such as, but not limited to, a dog or a cat, livestock, such as, but not limited to, a cow, a horse, or a pig, a primate, or a human. Subject also includes model organisms, including, for example, *Drosophila*, the nematode *C. elegans*, or animal models used, for example, for the study of NaCT structure or function, life span and weight gain. A “non-human animal” refers to any animal that is not a human and includes vertebrates such as rodents, non-human primates.

A “control” sample or subject is one in which a NaCT polypeptide has not been manipulated in any way.

As used herein *in vitro* is in cell culture, *ex vivo* is a cell that has been removed from the body of a subject and *in vivo* is within the body of a subject.

Unless otherwise specified, “a,” “an,” “the,” and “at least one” are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The full-length cDNA nucleotide sequence (SEQ ID NO:1) and translated amino acid sequence (SEQ ID NO:2) of *D. melanogaster* Indy.

Figure 2. Comparison of succinate uptake by drIndy (Fig. 2A) and hNADC3 (Fig. 2B) in the presence or absence of Na⁺. HRPE cells were transfected with vector alone, drIndy cDNA or hNADC3 cDNA. Uptake of succinate (40 nM) was measured in the presence of either NaCl (+Na⁺) or *N*-methyl-D-glucamine chloride (-Na⁺). Data (means ± S.E.M.) are from nine independent measurements.

Figure 3. Saturation kinetics of succinate uptake via drIndy measured in the presence of Na^+ . Data (means \pm S.E.M.) represent only the cDNA-specific uptake and are from four independent measurements. Inset: Eadie-Hofstee plot [succinate uptake/succinate concentration (v/s) versus succinate uptake (v)].

5 Figure 4. Comparison of the ability of drIndy and hNaDC3 to transport citrate and pyruvate. Uptake of citrate (35 μM) (Fig. 4A) or pyruvate (135 μM) (Fig. 4B) was measured in the presence of NaCl in HRPE cells transfected with vector alone, drIndy cDNA, or hNaDC3 cDNA. Data (means \pm S.E.M.) are from three independent measurements.

10 Figure 5. Comparison of affinity for citrate for the transport process mediated by drIndy and hNaDC3. HRPE cells were transfected with vector alone, drIndy cDNA (●) or hNaDC3 cDNA (○). Uptake of succinate (80 nM) was measured in the presence of NaCl with or without increasing concentrations of citrate. Data (means \pm S.E.M.) represent only the cDNA-specific uptake and
15 are from three independent measurements.

Figure 6. Transport characteristics of drIndy and hNaDC3 in *X. laevis* oocytes. drIndy and hNaDC3 were expressed functionally in oocytes by injection of the respective cRNA. Water-injected oocytes served as control (Fig. 6A and Fig. 6B). Uptake of succinate (0.1 μM) was measured in the
20 presence of either NaCl ($+\text{Na}^+$) or choline chloride ($-\text{Na}^+$). Data (means \pm S.E.M.) are from ten oocytes (Fig. 6C and Fig. 6D). Succinate (2 mM)-induced inward currents were monitored using the two-micro-electrode voltage-clamp method in drIndy- and hNaDC3-expressing oocytes or in water-injected oocytes. The membrane potential was maintained at -50 mV. Perfusion buffers
25 contained either NaCl ($+\text{Na}^+$) or choline chloride ($-\text{Na}^+$).

Figure 7. The full-length cDNA nucleotide sequence (SEQ ID NO:3) and translated amino acid sequence (SEQ ID NO:4) of rat NaCT transporter.

Figure 8. Alignment of amino acid sequence of rat NaCT (SEQ ID NO:4) with that of rat NaDC1 (SEQ ID NO:13) and rat NaDC3 (SEQ ID
30 NO:14). Regions of similarity are shaded.

Figure 9. Tissue expression pattern of NaCT mRNA in rat by Northern analysis. A commercially available rat multiple tissue blot was hybridized sequentially first with a rat NaCT-specific probe and then with a rat β -actin-

specific probe under high stringency conditions. Lanes 1–12 present brain, thymus, lung, heart, skeletal muscle, stomach, small intestine, liver, kidney, spleen, testis, and skin, respectively.

Figure 10. Uptake of citrate, succinate, and pyruvate by rat NaCT. Fig. 10A shows the uptake of [^3H]succinate (80 nM), [^{14}C]pyruvate (135 μM), and [^{14}C]citrate (35 μM) in vector-transfected (*V*) and rat NaCT cDNA-transfected (NaCT) HRPE cells. Fig. 10B is a time course of [^{14}C]citrate (18 μM) uptake in vector-transfected (\circ) and rat NaCT cDNA-transfected (\bullet) HRPE cells. Fig. 10C shows the influence of extracellular pH on the uptake of [^{14}C]citrate (7 μM) that was mediated specifically via rat NaCT.

Figure 11. Influence of substrate concentration, sodium concentration, and membrane potential on citrate uptake mediated by rat NaCT. Fig. 11A shows saturation kinetics of citrate uptake via rat NaCT. Inset shows Eadie-Hofstee plot (v , citrate uptake in pmol/ 10^6 cells/minute; s , citrate concentration in μM). Fig. 11B shows the dependence of rat NaCT-mediated citrate (7 μM) uptake on Na^+ concentration. Fig. 11C shows uptake of citrate (20 μM) by rat NaCT under normal (5 mM K^+) and membrane depolarizing (55 mM K^+) conditions.

Figure 12. Substrate selectivity of rat NaCT and NaDC3. Fig. 12A shows inhibition of rat NaCT-mediated [^{14}C]citrate (14 μM) uptake by increasing concentrations of citrate (\bullet), succinate (\circ), cis-aconitate (\blacktriangledown), fumarate (\square), α -ketoglutarate (Δ), and isocitrate (\blacksquare). Uptake measured in the absence of inhibitors was taken as 100%. Fig. 12B shows inhibition of rat NaDC3-mediated [^3H]succinate (80 nM) uptake by increasing concentrations of succinate (\circ), fumarate, (\square), α -ketoglutarate (Δ), citrate (\bullet), isocitrate (\blacksquare), and cis-aconitate (\blacktriangledown). Uptake measured in the absence of inhibitors was taken as 100%.

Figure 13. Analysis of expression pattern of NaCT mRNA in mouse brain by *in situ* hybridization. Fig. 13A, hybridization with an antisense riboprobe specific for mouse NaCT. CC, cerebral cortex; OB, olfactory bulb; HCF, hippocampal formation; CB, cerebellum. Fig. 13B, hybridization with a sense riboprobe specific for mouse NaCT (negative control). Fig. 13C and Fig. 13D, higher power magnification of cerebellar region hybridized with the

antisense probe. M, molecular layer with stellate and basket cells; P, Purkinje cell layer; G, granular layer; W, white matter; DCN, deep cerebellar nuclei.

Fig. 13E and Fig. 13F, higher power magnification of hippocampal formation region hybridized with the antisense probe. M, molecular layer of dentate gyrus; G, granulate layer of dentate gyrus; PM, polymorphic layer of dentate gyrus; CA, cornu ammonis neurons; S, subiculum.

Figure 14. The full-length cDNA nucleotide sequence (SEQ ID NO:5) and translated amino acid sequence (SEQ ID NO:5) of human NaCT transporter.

Figure 15. Alignment of amino acid sequence of human NaCT (SEQ ID NO:6) with that of rat NaCT (SEQ ID NO:4). Regions of sequence similarity are shaded.

Figure 16. Exon-intron organization of the human *nact* gene. Exons are numbered in bold in the gene. The other numbers in the gene show the relative positions of the exons and introns in the approximately 30 kb gene. The shaded areas in exon 1 and exon 12 denote the 5'- and 3'-untranslated regions. Numbers in the cDNA indicate the nucleotide positions of the splice junctions. The exact length of the first exon is not known because of lack of information on the transcription start site.

Figure 17. Transport of monocarboxylates, dicarboxylates, and tricarboxylates by NaCT. Figure 17A represents Relative abilities of human NaCT to transport monocarboxylates, dicarboxylates, and tricarboxylates. Uptake of [14 C]-pyruvate (100 μ M), [3 H]-succinate (80 nM), and [14 C]-citrate (20 μ M) was measured in vector-transfected (V) and human NaCT cDNA-transfected (NaCT) HRPE cells. Figure 17B is a time course of citrate uptake mediated by human NaCT. Uptake of [14 C]-citrate (20 μ M) was measured in vector-transfected (○) and human NaCT cDNA-transfected (●) HRPE cells.

Figure 18. Kinetics of citrate transport. Figure 18A shows saturation kinetics of citrate uptake via human NaCT. Inset: Eadie-Hofstee plot (v, citrate uptake in nmol/ 10^6 cells/min; s, citrate concentration in mM). Figure 18B shows dependence of human NaCT-mediated citrate (20 μ M) uptake on Na^+ concentration.

Figure 19. The full-length cDNA nucleotide sequence (SEQ ID NO:7) and translated amino acid sequence (SEQ ID NO:8) of *C. elegans* NaCT.

Figure 20. Structure of the *C. elegans nact* gene. Exons are indicated by filled boxes and numbered accordingly; introns, by solid lines. The untranslated regions in exon 1 and exon 11 are indicated by blank boxes. The consensus polyadenylation signal AATAAA is also shown. Sizes and positions of the exons and the introns are drawn to the exact scale.

Figure 21. Amino acid sequence similarity among NaCTs from *Drosophila* (SEQ ID NO:2), *C. elegans* (SEQ ID NO:8), and rat (SEQ ID NO:4).

Figure 22. Functional characteristics of *C. elegans* NaCT in a mammalian cell expression system. Fig. 22A is a comparison of the transport activities of NaDC1, NaDC2 and NaCT from *C. elegans*. Uptake of 10 μ M citrate or 10 μ M succinate was measured in HRPE cells transfected with the transporter cDNAs. Uptake measured in the vector (pSPORT)-transfected cells served as a control for endogenous uptake activity. Values (cDNA-specific activity) represent means \pm S.E. for four determinations. Fig. 22B shows ion-dependence of *C. elegans* NaCT-mediated citrate uptake in HRPE cells. Uptake of 10 μ M citrate was measured in buffers containing 140 mM Na⁺, Li⁺, K⁺, and NMDG (as chloride salts), or 300 mM mannitol. Values represent means \pm S.E. for four determinations. Fig. 22C shows substrate specificity of ceNaCT-mediated uptake. Uptake of 10 μ M [¹⁴C]citrate was measured in the absence or presence of potential inhibitors (2.5 mM) in cells transfected with vector alone or ceNaCT cDNA. The cDNA-specific uptake was calculated by adjusting for the uptake in vector-transfected cells. The cDNA-specific uptake in the absence of inhibitors was taken as the control (100%) and the uptake in the presence of inhibitors is given as percent of this control value. Fig. 22D shows influence of extracellular pH on *C. elegans* NaCT-mediated citrate or succinate (10 μ M) uptake in HRPE cells.

Figure 23. Saturation kinetics of citrate and succinate uptake mediated by *C. elegans* NaCT in HRPE cells. Uptake of citrate (Fig. 23A) or succinate (Fig. 23B) was measured in a NaCl-containing medium (pH 7.5) over a substrate concentration range of 10-1000 μ M in cells transfected with vector or

C. elegans NaCT cDNA. The cDNA-specific uptake was calculated by adjusting for the endogenous uptake measured in vector-transfected cells. Values represent means \pm S.E. for four determinations. Insets are the Eadie-Hofstee transformation of the data.

5 Figure 24. Functional characteristics of *C. elegans* NaCT in *Xenopus* oocyte expression system. In Fig. 24A uptake of [14 C]-citrate (40 μ M) was measured in control (water-injected) oocytes and in oocytes injected with *C. elegans* NaCT cRNA at pH 6.5 in the presence of NaCl. Values represent mean \pm S.E ($n=8-10$ oocytes). Fig. 24B shows ion-dependence of the citrate-evoked
10 currents under voltage-clamp conditions in oocytes expressing *C. elegans* NaCT. Oocytes were sequentially superfused with 250 μ M of citrate in a Na $^{+}$ -containing buffer (NaCl), or in a chloride-free buffer (NaGlu) in which NaCl was replaced isoosmotically with sodium gluconate or in a Na $^{+}$ -free buffer (CholineCi) in which NaCl was replaced isoosmotically with choline chloride.
15 Fig. 24C shows the effects of pH on substrate (250 μ M)-induced currents in oocytes expressing *C. elegans* NaCT. Fig. 24D is a kinetic analyses of citrate-evoked inward currents in oocytes expressing *C. elegans* NaCT at different testing membrane potentials. The perfusion buffer (pH 6.5) contained NaCl.

 Figure 25. Effect of the knockdown of NaCT by RNAi on life span and
20 body size in *C. elegans*. Fig. 25A shows the effect of the knockdown of NaCT by RNAi on life span in *C. elegans*. The knockdown of NaCT was done by feeding the worms with bacteria producing NaCT-specific dsRNA. The knockdown of DAF-2 was included as a positive control. Worms fed on bacteria carrying the empty vector pPD129 served as the wild type control. The
25 curves show the survival probability of the worms in different experimental groups at a given day after hatching under the influence of the gene-specific dsRNAs. Fig. 25B is a graphical representation of the effect of the knockdown of NaCT by RNAi on body size in *C. elegans*. Results from gene-specific RNAi for ceNaDC1 and ceNaDC2 have been included.

30 Figure 26. Effect of NaCT knockdown on fat deposition in *C. elegans*. Comparison of the fluorescence intensity of the Nile red staining between worms with NaCT knockdown by RNAi (hatched bar, N=13) and control worms (empty bar, N=80). The intensity in control worms was taken as 1.

Figure 27. The full-length cDNA nucleotide sequence (SEQ ID NO:9) and translated amino acid sequence (SEQ ID NO:10) of mouse NaCT.

Figure 28. Amino acid sequence of mouse NaCT and the exon-intron organization of murine *nact* gene. Fig. 28A shows a comparison of the primary structure of mouse NaCT (SEQ ID NO:10) with that of rat (SEQ ID NO:4) and human (SEQ ID NO:6) NaCTs. Identical amino acids are indicated by dark shading and conserved amino acid substitutions are indicated by light shading. In Fig. 28B, exons, identified by boxes, are numbered in the gene and numbers above the exon boxes indicate the number of base pairs in respective exons.

The numbers associated with the introns indicate the size of the respective introns in kilobase pairs. The shaded region in exon 12 denotes the 3'-untranslated region.

Figure 29. Uptake of succinate and citrate via mouse NaCT. HRPE cells were transfected with either vector alone (C) or mouse NaCT cDNA (NaCT). Uptake of [³H]-succinate (50 nM) and [¹⁴C]-citrate (20 μM) was measured in transfected cells. The uptake of each substrate measured in control cells was taken as 1 and the corresponding uptake in cDNA-transfected cells is given as a ratio (–fold increase) in comparison with this control uptake.

Figure 30. Kinetics of mouse NaCT-mediated citrate and succinate uptake. HRPE cells were transfected with either vector alone or mouse NaCT cDNA and uptake of citrate (Fig. 30A) and succinate (Fig. 30B) was measured in these cells. The concentration range was 5-250 μM for citrate and 2.5-1000 μM for succinate. The uptake measured in vector-transfected cells was subtracted from the corresponding uptake measured in cDNA-transfected cells to determine the cDNA-specific uptake. Only uptake values that are specific for mouse NaCT were used in kinetic analysis. Insets, Eadie-Hosftee plots: V/S (uptake rate/substrate concentration) versus V (uptake rate).

Figure 31. Na⁺-activation kinetics of citrate and succinate uptake mediated by mouse NaCT. HRPE cells were transfected with either vector alone or mouse NaCT cDNA and uptake of citrate (20 μM) (Fig. 31A) and succinate (2.5 μM) (Fig. 31B) was measured in these cells. Concentration of Na⁺ was varied over the range of 10-140 mM by adjusting the concentrations of NaCl and *N*-methyl-D-glucamine chloride appropriately to maintain the

osmolality. The uptake measured in vector-transfected cells was subtracted from the corresponding uptake measured in cDNA-transfected cells to determine the cDNA-specific uptake. Only the uptake values specific for mouse NaCT were used in kinetic analysis.

5 Figure 32. Influence of extracellular pH on mouse NaCT-mediated uptake of citrate and succinate. HRPE cells were transfected with either vector alone or mouse NaCT cDNA and uptake of citrate (10 μ M) (●) and succinate (10 μ M) (○) was measured in these cells. The pH of the uptake buffer was varied by appropriately adjusting the concentrations of Mes, Hepes, and Tris.

10 The uptake measured in vector-transfected cells was subtracted from the corresponding uptake measured in cDNA-transfected cells to determine the cDNA-specific uptake. Data represent only the cDNA-specific uptake.

 Figure 33. Electrogenicity of mouse and rat NaCTs. Mouse and rat NaCTs were expressed functionally in *X. laevis* oocytes by injection of

15 respective cRNAs. Citrate-induced currents were monitored in these oocytes using the two-microelectrode voltage-clamp technique. The membrane potential was clamped at -50 mV. The perfusion buffer contained *N*-methyl-D-glucamine chloride ($-\text{Na}^+$), NaCl, or sodium gluconate ($-\text{Cl}^-$).

 Figure 34. Relative ability of rat NaCT to transport various citric acid cycle intermediates and other related compounds. Rat NaCT was expressed

20 functionally in *X. laevis* oocytes by injection of cRNA. The oocytes were perfused with various monocarboxylates, dicarboxylates, and tricarboxylates (0.5 mM) and the substrate-induced inward currents were monitored using the two-microelectrode voltage-clamp technique. The currents induced by various

25 substrates are given as percent of the current induced by citrate. The data are from three different oocytes and the citrate-induced current in each oocyte was normalized by taking this value as 100%. The value for citrate-induced current in three different oocytes was 87 ± 8 nA.

 Figure 35. Determination of charge-to-substrate ratio for rat NaCT with

30 citrate and succinate as substrates. Rat NaCT was expressed functionally in *X. laevis* oocytes by injection of cRNA. The oocytes were perfused with 50 μ M citrate or succinate (radiolabeled plus unlabeled substrates) for 10 minutes and the substrate-induced currents were monitored in these oocytes using the two-

microelectrode voltage-clamp technique. The membrane potential was clamped at -50 mV. At the end of the experiment, the oocytes were washed with the perfusion buffer and the radioactivity associated with the oocytes was determined. The quantity of charge transferred into the oocytes during

5 perfusion with the substrates was determined from the integration of the area covered by the time *versus* inward current curves and the quantity of the substrates transferred into the oocytes was determined from the radioactivity associated with the oocytes. Fig. 35A shows the relationship between substrate uptake and charge transfer for citrate and succinate in three different oocytes.

10 Fig. 35B shows the charge-to-substrate ratio for citrate and succinate.

Figure 36. The full-length cDNA nucleotide sequence (SEQ ID NO:11) and translated amino acid sequence (SEQ ID NO:12) of zebrafish NaCT.

Figure 37. Comparison of the amino acid sequence of zebrafish NaCT (SEQ ID NO:12) with that of rat (SEQ ID NO:4), mouse (SEQ ID NO:10), and

15 human (SEQ ID NO:6).

Figure 38. Citrate uptake by cells transfected with zebrafish NaCT. Fig. 38A shows a time course of citrate ($2\text{ }\mu\text{M}$) uptake in cells transfected with either vector alone (○) or zebrafish NaCT cDNA (●). Fig. 38B demonstrates the influence of pH on citrate ($1\text{ }\mu\text{M}$) uptake mediated by zebrafish NaCT.

20 Figure 39. Saturation kinetics (Fig. 39A) and Na^+ -activation kinetics (Fig. 39B) of citrate uptake mediated by zebrafish NaCT. The Michaelis constant for citrate uptake is $40 \pm 4\text{ }\mu\text{M}$. The value for Hill coefficient for the activation of uptake is 26 ± 0.2 .

Figure 40. Inhibition of citrate uptake. Fig. 40A shows the inhibition of

25 zebrafish NaCT-mediated [^{14}C]-citrate ($1\text{ }\mu\text{M}$) uptake by various structural analogs (2mM). Fig. 40B demonstrates dose-response relationships for inhibition of zebrafish NaCT-mediated [^{14}C]-citrate ($1\text{ }\mu\text{M}$) uptake by citrate (●), succinate (○), and cis-aconitate (▼). The IC_{50} values for the inhibition are 30 ± 4 , 51 ± 9 , and $624 \pm 45\text{ }\mu\text{M}$, respectively, for citrate, succinate, and

30 cis-aconitate.

Figure 41. Differential effect of Li^+ on the uptake of citrate ($20\text{ }\mu\text{M}$) via rat NaCT () and human NaCT (○).

Figure 42. Substrate saturation kinetics and Na⁺-activation kinetics for human NaCT. Fig. 42A shows substrate saturation kinetics of human NaCT in the absence (□) and presence (□) of 10 mM Li⁺. Fig. 42B shows Na⁺-activation kinetics of human NaCT in the absence (□) and presence (□) of 10 mM Li⁺.

Figure 43. Incorporation of citrate and acetate into lipids in HepG2 cells in the absence and presence of Li⁺. Fig. 43A is a histogram showing the incorporation of [¹⁴C]citrate in HepG2 cells in the absence of Li⁺, or in the presence of 2 or 10 mM Li⁺. Fig. 43B is a histogram showing the incorporation of [¹⁴C]acetate into lipids for the same concentrations of Li⁺.

Figure 44. Structure-function relationship for NaCT. Fig. 44A demonstrates the influence of Li⁺ (10 mM) on the uptake of citrate (20 μM) via wild type human and rat NaCTs and the chimeric transporter in which the region containing the amino acids 496-516 in human NaCT has been replaced with the corresponding region from rat NaCT. Fig. 44B compares the amino acid sequences between human NaCT (amino acids 496-516) and rat NaCT (amino acids 500-520). The amino acids that are different between human and rat NaCTs are identified in bold. Fig. 44C shows substrate saturation kinetics of wild type human NaCT (○) and the Phe→Leu mutant of human NaCT (○). Fig. 44D demonstrates the influence of increasing concentrations of Li⁺ on the uptake of citrate (20 μM) via wild type human NaCT (○) and the Phe→Leu mutant of human NaCT (○).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

With the present invention, a new transmembrane citrate transporter has been identified and functionally characterized. This transporter efficiently transports citrate. As citrate transport by this transporter is Na⁺-couple, this transmembrane transporter is herein designated as “NaCT” for “Na⁺-coupled citrate transporter.” The NaCT polypeptide of the present invention is involved in the utilization of extracellular citrate for the synthesis of fatty acids and cholesterol. The NaCT polypeptide of the present invention will serve as a drug target for the treatment of obesity, hyperlipidemia, and hypercholesterolemia.

The present invention also provides the functional characterization of *Drosophila* Indy (DrIndy) as a citrate transporter and identifies DrIndy as the *Drosophila* ortholog of mammalian NaCT.

5 *Polypeptides:*

As used herein, a “NaCT polypeptide” demonstrates one or more of the functional activities of a Na⁺-coupled transmembrane citrate transporter. Each of these functional activities of a NaCT polypeptide, and the assays for measuring these functional activities, are described in more detail herein.

- 10 Briefly, the functional activities of a NaCT polypeptide include, but are not limited to, one or more of the following. A NaCT polypeptide may demonstrate the transmembrane transport of citrate. A NaCT polypeptide may demonstrate Na⁺-dependent transmembrane transport of citrate. A NaCT polypeptide may demonstrate Na⁺-dependent transmembrane transport of citrate that is
- 15 modulated by Li⁺. Such modulation includes, but is not limited to, the stimulation of citrate transport and the inhibition of citrate transport. The Na⁺-dependent transmembrane transport of citrate by a NaCT polypeptide may demonstrate a requirement for multiple Na⁺ ions for transport coupling. The stoichiometry of this coupling may be, for example, 2:1, 3:1, 4:1, or 5:1. A
- 20 NaCT polypeptide may demonstrate transmembrane transport of citrate that is electrogenic.

A NaCT polypeptide may include a sodium symporter family signature motif. The consensus pattern for such a sodium symporter family signature motif is: (S)SXXFXXP(V)(G)XXXNX(I)V (SEQ ID NO:29), wherein X

- 25 denotes any amino acid residue, (S) denotes serine or other related amino acids, such as alanine, cysteine, threonine, or proline, (V) denotes valine or other related amino acids, such as leucine, isoleucine or methionine, (G) denotes glycine or other related amino acids, such as serine or alanine, and (I) denotes isoleucine or other related amino acids, such as leucine, valine, or methionine.
- 30 The sodium symporter family is a group of integral membrane proteins that mediate the cellular uptake of a wide variety of molecules including di- or tri-carboxylates and sulfate by a transport mechanism involving sodium cotransport (Pajor, *Annu Rev Physiol* (1999);61: 663-682 and Pajor, *J Membr*

Biol (2000);175 :1-8)).

As used herein, a “citrate transporter polypeptide” demonstrates transmembrane transport of citrate. The transmembrane transport of citrate by a citrate transporter polypeptide need not be Na⁺-coupled.

5 The NaCT polypeptides of the present invention may be derived from a variety of species, including, but not limited to, human, primate, rat, mouse, *C. elegans*, and zebrafish. For example, the NaCT polypeptides of the present invention include, but are not limited to, rat NaCT (SEQ ID NO:4), human NaCT (SEQ ID NO:6), *C. elegans* NaCT (SEQ ID NO:8), mouse NaCT (SEQ
10 ID NO:10), and zebrafish NaCT (SEQ ID NO:12). A citrate transporter polypeptide of the present invention may be derived from a variety of species. One example of a citrate transporter polypeptide is *Drosophila* Indy (DrIndy), having SEQ ID NO:2, as described in more detail in Example 1.

 The polypeptides of the present invention also include “biologically
15 active analogs” of naturally occurring polypeptides. For example, the NaCT polypeptides of the present invention include, but are not limited to, biologically active analogs of rat NaCT (SEQ ID NO:4), human NaCT (SEQ ID NO:6), *C. elegans* NaCT (SEQ ID NO:8), mouse NaCT (SEQ ID NO:10), and zebrafish NaCT (SEQ ID NO:12). The citrate transport polypeptides of the present
20 invention includes, but is not limited to, biologically active analogs of DrIndy (SEQ ID NO:2).

 As used herein, a “biologically active analog” demonstrates one or more of the following functional activities; demonstrate the transmembrane transport of citrate; demonstrate Na⁺-dependent transmembrane transport of citrate;
25 demonstrate Na⁺-dependent transmembrane transport of citrate that is modulated by Li⁺, with such modulation including, but is not limited to, the stimulation of citrate transport and the inhibition of citrate transport; demonstrate a requirement for multiple Na⁺ ions for transport coupling, where the stoichiometry of this coupling may be, for example, 2:1, 3:1, 4:1, or 5:1; and
30 demonstrate transmembrane transport of citrate that is electrogenic. Functional activity of a NaCT polypeptide can be easily assessed using the various assays described herein as well as other assays well known to one with ordinary skill in the art. A modulation in functional activity, including the stimulation or the

inhibition of functional activity, can be readily ascertained by the various assays described herein, and by assays known to one of skill in the art.

A modulation in a functional activity can be quantitatively measured and described as a percentage of the functional activity of a comparable control.

5 The functional activity of the present invention includes a modulation that is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 110%, at least 125%, at least
10 150%, at least 200%, or at least 250% of the activity of a suitable control.

For example, the stimulation of a functional activity can be quantitatively measured and described as a percentage of the functional activity of a comparable control. The functional activity of the present invention includes a stimulation that is at least 5%, at least 10%, at least 15%, at least
15 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 110%, at least 125%, at least 150%, at least 200%, or at least 250% of the activity of a suitable control.

20 For example, inhibition of a functional activity can be quantitatively measured and described as a percentage of the functional activity of a comparable control. The functional activity of the present invention includes an inhibition that is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least
25 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 110%, at least 125%, at least 150%, at least 200%, or at least 250% of the activity of a suitable control.

A “biologically active analog” of a polypeptide includes polypeptides
30 having one or more amino acid substitutions that do not eliminate a functional activity. Substitutes for an amino acid in the polypeptides of the invention may be selected from other members of the class to which the amino acid belongs. For example, it is well-known in the art of protein biochemistry that an amino

acid belonging to a grouping of amino acids having a particular size or characteristic (such as charge, hydrophobicity and hydrophilicity) can be substituted for another amino acid without altering the activity of a protein, particularly in regions of the protein that are not directly associated with biological activity. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of such preferred conservative substitutions include Lys for Arg and vice versa to maintain a positive charge; Glu for Asp and vice versa to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH₂. Likewise, biologically active analogs of a NaCT polypeptide containing deletions or additions of one or more contiguous or noncontiguous amino acids that do not eliminate a functional activity of a NaCT polypeptide are also contemplated.

A "biologically active analog" of a NaCT polypeptide includes "fragments" and "modifications" of a NaCT polypeptide. As used herein, a "fragment" of a NaCT polypeptide means a NaCT polypeptide that has been truncated at the N-terminus, the C-terminus, or both. A fragment may range from about 5 to about 250 amino acids in length. For example it may be about 5, about 10, about 20, about 25, about 50, about 75, about 100, about 125, about 150, about 175, about 200, about 225, or about 250 amino acids in length. Fragments of a NaCT polypeptide with potential biological activity can be identified by many means. One means of identifying such fragments of a NaCT polypeptide with biological activity is to compare the amino acid sequences of a NaCT polypeptide from rat, mouse, human and/or other species to one another. Regions of homology can then be prepared as fragments. Fragments of a polypeptide also include a portion of the polypeptide containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the

resulting polypeptide still retains a biological activity of the full-length polypeptide.

A “modification” of a NaCT polypeptide includes NaCT polypeptides or fragments thereof chemically or enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like. Modified polypeptides of the invention may retain the biological activity of the unmodified polypeptide or may exhibit a reduced or increased biological activity.

The polypeptides and biologically active analogs thereof of the present invention include native (naturally occurring), recombinant, and chemically or enzymatically synthesized polypeptides. For example, the NaCT polypeptides of the present invention may be prepared by isolation from naturally occurring tissues or prepared recombinantly, by well known methods, including, for example, preparation as fusion proteins in bacteria and insect cells.

The polypeptides of the present invention include polypeptides with “structural similarity” to naturally occurring polypeptides, such as *Drosophila* drIndy (SEQ ID NO:2), rat NaCT (SEQ ID NO:4), human NaCT (SEQ ID NO:6), *C. elegans* NaCT (also referred to as CeNaCT) (SEQ ID NO:8), mouse NaCT (SEQ ID NO:10), or zebrafish NaCT (SEQ ID NO:12).

As used herein, “structural similarity” refers to the identity between two polypeptides. For polypeptides, structural similarity is generally determined by aligning the residues of the two polypeptides (for example, a candidate polypeptide and the polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12) to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. A candidate polypeptide is the polypeptide being compared to the polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID

NO:12. A candidate polypeptide can be isolated, for example, from an animal or a microbe, or can be produced using recombinant techniques, or chemically or enzymatically synthesized.

5 A pair-wise comparison analysis of transporter protein sequences can be carried out using the BESTFIT algorithm in the GCG package (version 10.2, Madison WI). Alternatively, polypeptides may be compared using the Blastp program of the BLAST 2 search algorithm, as described by Tatiana et al., (*FEMS Microbiol Lett*, 174, 247-250 (1999)), and available on the world wide web at ncbi.nlm.nih.gov/BLAST/. The default values for all BLAST 2 search
10 parameters may be used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and filter on.

In the comparison of two amino acid sequences, structural similarity may be referred to by percent “identity” or may be referred to by percent
15 “similarity.” “Identity” refers to the presence of identical amino acids and “similarity” refers to the presence of not only identical amino acids but also the presence of conservative substitutions.

The NaCT polypeptides of the present invention include polypeptides with at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least
20 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence similarity to a known NaCT polypeptide, including, but not limited to, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

The NaCT polypeptides of the present invention also include
25 polypeptides with at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to a known NaCT polypeptide, including, but not limited to, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

30 Some structural similarities between NaCT polypeptides of the present invention are as follows. Rat NaCT (SEQ ID NO:4) compared to rat NaDC1 (SEQ ID NO:13) demonstrates 62% amino acid sequence similarity and 50% amino acid sequence identity. Rat NaCT (SEQ ID NO:4) compared to rat

NaDC3 (SEQ ID NO:14) demonstrates 59% amino acid sequence similarity and 48% amino acid sequence identity.

CeNaCT (SEQ ID NO:8) compared to DrIndy (SEQ ID NO:2) demonstrates 48% amino acid sequence similarity and 35% amino acid sequence identity. CeNaCT (SEQ ID NO:8) compared to rat NaCT (SEQ ID NO:4) demonstrates 49% amino acid sequence similarity and 36% amino acid sequence identity. CeNaCT (SEQ ID NO:8) compared to mouse NaCT (SEQ ID NO:10) demonstrates 48% amino acid sequence similarity and 35% amino acid sequence identity. CeNaCT (SEQ ID NO:8) compared to human NaCT (SEQ ID NO:6) demonstrates 49% amino acid sequence similarity and 37% amino acid sequence identity.

DrIndy (SEQ ID NO:2) compared to rat NaCT (SEQ ID NO:4) demonstrates 51% amino acid sequence similarity and 37% amino acid sequence identity. DrIndy (SEQ ID NO:2) compared to mouse NaCT (SEQ ID NO:10) demonstrates 49% amino acid sequence similarity and 36% amino acid sequence identity. DrIndy (SEQ ID NO:2) compared to human NaCT (SEQ ID NO:6) demonstrates 52% amino acid sequence similarity and 40% amino acid sequence identity. DrIndy (SEQ ID NO:2) compared to human NaDC1 (Genbank Accession No. 26209) demonstrates 35% amino acid sequence identity. DrIndy (SEQ ID NO:2) compared to human NaDC3 (Genbank Accession No. AF154121) demonstrates 34% amino acid sequence identity.

Human NaCT (SEQ ID NO:6) compared to rat NaCT (SEQ ID NO:4) demonstrates 87% amino acid sequence similarity and 77% amino acid sequence identity. Human NaCT (SEQ ID NO:6) compared to human Na⁺-coupled sulfate transporter NaSi (GenBank Accession No. AF260824) demonstrates 43% amino acid sequence identity. Human NaCT (SEQ ID NO:6) compared to human sulfate transporter SUT-1 (GenBank Accession No. AF169301) demonstrates 40% amino acid sequence identity.

Mouse NaCT (SEQ ID NO:10) compared to rat NaCT (SEQ ID NO:4) demonstrates 93% amino acid sequence similarity and 86% amino acid sequence identity. Mouse NaCT (SEQ ID NO:10) compared to human NaCT (SEQ ID NO:6) demonstrates 85% amino acid sequence similarity and 74% amino acid sequence identity. Mouse NaCT (SEQ ID NO:10) demonstrates

50% amino acid sequence identity compared to mouse NaDC1; 44% amino acid sequence identity compared to mouse NaDC3; 40% amino acid sequence identity compared to mouse Na⁺-coupled sulfate transporter NaSi1; and 39% amino acid sequence identity compared to the mouse sulfate transporter SUT1.

5 Zebrafish NaCT (SEQ ID NO:12) compared to rat NaCT (SEQ ID NO:4) demonstrates 72% amino acid sequence similarity and 57% amino acid sequence identity. Zebrafish NaCT (SEQ ID NO:12) compared to human NaCT (SEQ ID NO:6) demonstrates 77% amino acid sequence similarity and 61% amino acid sequence identity. Zebrafish NaCT (SEQ ID NO:12)
10 compared to mouse NaCT (SEQ ID NO:10) demonstrates 74% amino acid sequence similarity and 57% amino acid sequence identity.

 The polypeptides of the present invention can also be designed to provide additional sequences, such as, for example, the addition of coding sequences for added C-terminal or N-terminal amino acids that would facilitate
15 purification by trapping on columns or use of antibodies. Such tags include, for example, histidine-rich tags that allow purification of polypeptides on nickel columns. Such gene modification techniques and suitable additional sequences are well known in the molecular biology arts.

 Amino acids essential for the function of NaCT polypeptides can be
20 identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244: 1081-1085, 1989; Bass et al., *Proc. Natl. Acad. Sci. USA* 88: 4498-4502, 1991).

 The polypeptides of the present invention may be formulated in a
25 composition along with a "carrier." As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as
30 any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

 By "pharmaceutically acceptable" is meant a material that is not

biologically or otherwise undesirable, i.e., the material may be administered to an individual along with a NaCT polypeptide without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

5

Polynucleotides:

The present invention provides isolated polynucleotides encoding NaCT polypeptides. As used herein a NaCT polypeptide is a polypeptide having one or more of the functional activities that are described herein. Examples of the present invention include an isolated polynucleotide having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, and the complements thereof. Also included in the present invention are polynucleotides hybridizing to one or more of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or a complement thereof, under standard hybridization conditions, that encode a polypeptide that exhibits one or more of the functional activities of a NaCT polypeptide. Also included in the present invention are polynucleotides having a sequence identity of at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% with the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11, where the polynucleotide encodes a polypeptide that exhibits one or more of the functional activities of a NaCT polypeptide.

As used herein, "sequence identity" refers to the identity between two polynucleotide sequences. Sequence identity is generally determined by aligning the residues of the two polynucleotides (for example, aligning the nucleotide sequence of the candidate sequence and the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11) to optimize the number of identical nucleotides along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of shared nucleotides, although the nucleotides in each sequence must nonetheless remain in their

proper order. A candidate sequence is the sequence being compared to a known sequence, such as SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11. For example, two polynucleotide sequences can be compared using the Blastn program of the BLAST 2 search algorithm, as
5 described by Tatiana et al., *FEMS Microbiol Lett.*, 1999;174: 247-250, and available on the world wide web at ncbi.nlm.nih.gov/BLAST/. The default values for all BLAST 2 search parameters may be used, including reward for match = 1, penalty for mismatch = -2, open gap penalty = 5, extension gap penalty = 2, gap x_dropoff = 50, expect = 10, wordsize = 11, and filter on.

10 In some aspects of the present invention, the polynucleotides of the present invention include nucleotide sequences having a sequence identity with SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11, or at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least
15 96%, at least 97%, at least 98%, or at least 99% identity with SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

Also included in the present invention are polynucleotide fragments. A polynucleotide fragment is a portion of an isolated polynucleotide as described herein. Such a portion may be several hundred nucleotides in length, for
20 example about 100, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900 or about 1000 nucleotides in length. Such a portion may be about 10 nucleotides to about 100 nucleotides in length, including but not limited to, about 14 to about 40 nucleotides in length.

The polynucleotides of the present invention may be formulated in a
25 composition along with a "carrier." As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as
30 any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

By "pharmaceutically acceptable" is meant a material that is not

biologically or otherwise undesirable, i.e., the material may be administered to an individual along with a NaCT polynucleotide without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Polynucleotides of the present invention can be inserted into a vector. Construction of vectors containing a polynucleotide of the invention employs standard ligation techniques known in the art. See, for instance, Sambrook et al, "Molecular Cloning: A Laboratory Manual," *Cold Spring Harbor Laboratory Press*, 1989. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, or artificial chromosome vectors. Typically, a vector is capable of replication in a bacterial host, for instance, *E. coli*. Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. A vector can provide for further cloning (amplification of the polynucleotide), e.g., a cloning vector, or for expression of the polypeptide encoded by the coding sequence, e.g., an expression vector. Suitable host cells for cloning or expressing the vectors herein are prokaryote or eukaryotic cells.

As used herein, an "expression vector" is a DNA molecule, linear or circular, that includes a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and optionally one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

By "host cell" is meant a cell that supports the replication or expression of an expression vector. Host cells may be bacterial cells, including, for example, *E. coli* and *B. subtilis*, or eukaryotic cells, such as yeast, including, for example, *Saccharomyces* and *Pichia*, insect cells, including, for example, *Drosophila* cells and the Sf9 host cells for the baculovirus expression vector, amphibian cells, including, for example, *Xenopus* oocytes and mammalian cells, such as CHO cells, HeLa cells, human retinal pigment epithelial (RPE) cells, human hepatoma HepG2 cells, and plant cells.

An expression vector optionally includes regulatory sequences operably linked to the coding sequence. The invention is not limited by the use of any particular promoter, and a wide variety of promoters are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding sequence. The promoter used can be a constitutive or an inducible promoter. It can be, but need not be, heterologous with respect to the host cell.

The transformation of a host cell with an expression vector may be accomplished by a variety of means known to the art, including, but not limited to, calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, biolistics (i.e., particle bombardment) and the like.

Transformation of a host cell may be stable or transient. The term “transient transformation” or “transiently transformed” refers to the introduction of one or more transgenes into a cell in the absence of integration of the transgene into the host cell's genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) that detects the presence of a polypeptide encoded by one or more of the transgenes. Alternatively, transient transformation may be detected by detecting the activity of the protein encoded by the transgene. The term “transient transformant” refers to a cell that has transiently incorporated one or more transgenes. In contrast, the term “stable transformation” or “stably transformed” refers to the introduction and integration of one or more transgenes into the genome of a cell. The term “stable transformant” refers to a cell that has stably integrated one or more transgenes into the genomic DNA. Thus, a stable transformant is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more transgenes, genomic DNA from the transient transformant does not contain a transgene. Methods for both transient and stable expression of coding regions are well known in the art.

Among the known methods for expressing transporter genes is expression in a *Xenopus* oocyte system. A cDNA encoding the open reading frame of a citrate transporter polypeptide or portions thereof can be

incorporated into commercially available bacterial expression plasmids such as the pGEM (Promega) or pBluescript (Stratagene) vectors or one of their derivatives. After amplifying the expression plasmid in bacterial (*E. coli*) cells the DNA is purified by standard methods. The incorporated transporter
5 sequences in the plasmid DNA are then transcribed in vitro according to standard protocols, such as transcription with SP6 or T7 RNA polymerase. The RNA thus prepared is injected into *Xenopus* oocytes where it is translated and the resulting transporter polypeptides are incorporated into the plasma membrane. The functional properties of these transporters can then be
10 investigated by electrophysiological, biochemical, pharmacological, and related methods.

The polynucleotides of the present invention may be inserted into a recombinant DNA vector for the production of products including, but not limited to, mRNA, antisense oligonucleotides, and polynucleotides for use in
15 RNA interference (RNAi) (see, for example, Cheng et al., *Mol Genet Metab.* (2003);80: 121-28). For example, for the production of mRNA, a cDNA comprising, for example, SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or a fragments thereof, may be inserted into a plasmid containing a promoter for either SP6 or T7 RNA polymerase.
20 The plasmid is cut with a restriction endonuclease to allow run-off transcription of the mRNA, and the RNA is produced by addition of the appropriate buffer, ribonucleotides, and polymerase. The RNA is isolated by conventional means such as ethanol precipitation. The mRNA can be capped or polyadenylated, for example, prior to injection into a cell such as a *Xenopus* oocyte, for expression.

25 The NaCT polypeptide transports citrate, an important metabolic intermediate with multiple metabolic functions, including, for example, lipid and cholesterol synthesis. Thus, the present invention also includes transgenic and knockout animal models, useful in studies to further understand the physiological functions of this transporter. Such animals may be constructed
30 using standard methods known in the art and as set forth, for example, in U.S. Pat. Nos. 5,614,396 5,487,992, 5,464,764, 5,387,742, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,384, 5,175,383, 4,873,191, and 4,736,866.

Antibodies:

Included in the present invention are antibodies that specifically bind to one or more of the polypeptides described herein. Such antibodies include, but are not limited to, antibodies that specifically bind to *Drosophila* Indy (SEQ ID NO:2), rat NaCT (SEQ ID NO:4), human NaCT (SEQ ID NO:6), *C. elegans* NaCT (SEQ ID NO:8), mouse NaCT (SEQ ID NO:10), zebrafish NaCT (SEQ ID NO:12) and variants thereof. Such antibodies include, but are not limited to, polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, humanized antibodies, chimeric antibodies, anti-idiotypic antibodies, single chain antibodies, and antigen-binding fragments thereof, such as F(ab')₂ and Fab proteolytic fragments and fragments produced from an Fab expression library. The term "polyclonal antibody" refers to an antibody produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an antibody produced from a single clone of plasma cells.

As used herein, "antibodies" or "antibody" refers to an immunoglobulin molecule or immunologically active antigen-binding portion thereof. In preferred embodiments, an antibody has at least one, and preferably two, heavy (H) chain variable regions (abbreviated herein as VH), and at least one and preferably two light (L) chain variable regions (abbreviated herein as VL). The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al., *J. Mol. Biol.* 1987;196: 901-917). Each VH and VL is composed of three CDR's and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The phrase "specifically binds" or "specifically immunoreactive with," when referring to an antibody, refers to a binding reaction that is determinative of the presence of a protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified

antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein

Antibodies of the present invention can be prepared using the intact polypeptide or fragments thereof as the immunizing agent. If a polypeptide fragment is used as an immunizing agent, a preferred fragment is about 15 to about 30 contiguous amino acids of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12. For example, contiguous amino acid fragments of about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, or about 32 amino acids may be used. The polypeptide fragment may be selected from a non-transmembrane domain of a NaCT polypeptide, for example, in an extracellular or intracellular loop. A preferred antibody binds to an extracellular epitope and alters the functional ability of the transporter to transport citrate. Such antibodies may be identified using any of the methods for assaying transporters described herein.

In addition to specifically binding to a citrate transporter polypeptide, the antibodies may have additional binding specificities. For example, an antibody may bind to the C terminus or the N terminus of a citrate transport polypeptide. Or, an antibody may be selected that demonstrates limited cross reactivity. For example, an antibody may bind to a human NaCT polypeptide, but not to a rat NaCT polypeptide or mouse NaCT polypeptide; or an antibody may bind to a NaCT polypeptide of a given species, such as human, rat, mouse, zebrafish, or *C. elegans*, but not bind to the NADC1, NADC2 or NADC3 polypeptides of the same species.

The preparation of polyclonal antibodies is well known. Polyclonal antibodies may be obtained by immunizing a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, hamsters, guinea pigs and rats as well as transgenic animals such as transgenic sheep,

cows, goats or pigs, with an immunogen. The resulting antibodies may be isolated from other proteins by using an affinity column having an Fc binding moiety, such as protein A, or the like.

Monoclonal antibodies can be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see, for example, Kohler and Milstein, *Eur. J. Immunol.* (1976);6: 511-519; J. Goding (1986) In "Monoclonal Antibodies: Principles and Practice," Academic Press, pp 59-103; and Harlow et al., *Antibodies: A Laboratory Manual*, page 726 (Cold Spring Harbor Pub. 1988). Monoclonal antibodies can be isolated and purified from hybridoma cultures by techniques well known in the art.

In some embodiments, the antibody can be recombinantly produced, for example, produced by phage display or by combinatorial methods. Phage display and combinatorial methods can be used to isolate recombinant antibodies that bind to a NaCT polypeptide or fragments thereof (see, for example, U.S. Pat. No. 5,223,409; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; WO 90/02809; Fuchs et al., *Bio/Technology* (1991);9: 1370-1372; Huse et al., *Science* (1989);246: 1275-1281; Griffiths et al., *EMBO J.* (1993);12: 725-734; Hawkins et al., *J Mol Biol* (1992);226: 889-896; Clackson et al., *Nature* (1991);352: 624-628; Gram et al., *PNAS* (1992);89:3576-3580; Garrad et al., *Bio/Technology* (1991);9: 1373-1377; Hoogenboom et al., *Nuc Acid Res* (1991);19: 4133-4137; and Barbas et al., *PNAS* (1991);88: 7978-7982). Such methods can be used to generate human monoclonal antibodies.

Human monoclonal antibodies can also be generated using transgenic mice carrying the human immunoglobulin genes rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, for example, WO 91/00906; WO 91/10741; WO 92/03918, Lonberg et al., *Nature* (1994);368: 856-859; Green et al., *Nature Genet.* (1994);7: 13-21; Morrison et al., *PNAS* (1994);81: 6851-6855; Tuailon et al., *PNAS* (1993);90:3720-3724; Bruggeman et al., *Eur J Immunol* (1991);21:1323-1326).

A therapeutically useful antibody may be derived from a “humanized” monoclonal antibody. Humanized monoclonal antibodies are produced by transferring one or more CDRs from the heavy and light variable chains of a mouse (or other species) immunoglobulin into a human variable domain, then substituting human residues into the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with immunogenicity of murine constant regions. Techniques for producing humanized monoclonal antibodies can be found, for example, in Jones et al., *Nature* (1986);321: 522 and Singer et al., *J. Immunol.*, (1993);150: 2844.

In addition, chimeric antibodies can be obtained by splicing the genes from a mouse antibody molecule with appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological specificity; see, for example, Takeda et al., *Nature* (1985);314: 544-546. A chimeric antibody is one in which different portions are derived from different animal species.

Antibody fragments can be generated by techniques well known in the art. Such fragments include Fab fragments produced by proteolytic digestion, and Fab fragments generated by reducing disulfide bridges.

Antibodies, or fragments thereof, may be coupled directly or indirectly to a detectable marker by techniques well known in the art. A detectable marker is an agent detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful detectable markers include fluorescent dyes, chemiluminescent compounds, radioisotopes, electron-dense reagents, enzymes, colored particles, biotin, or dioxigenin. A detectable marker often generates a measurable signal, such as radioactivity, fluorescent light, color, or enzyme activity.

When used for immunotherapy, antibodies, or fragments thereof, may be unlabelled or labeled with a therapeutic agent. These agents can be coupled directly or indirectly to the monoclonal antibody by techniques well known in the art, and include such agents as drugs, radioisotopes, lectins and toxins.

Antibodies can be used alone or in combination with additional therapeutic agents, such as those described above. Preferred combinations

include monoclonal antibodies with modifiers of citrate transporters or other biological response modifiers. The dosage administered may vary with age, condition, weight, sex, age and the extent of the condition to be treated, and can readily be determined by one skilled in the art. Dosages can be about 0.1 mg/kg
5 to about 2000 mg/kg. The monoclonal antibodies can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally, alone or with effector cells.

Antibodies that are both specific for a citrate transporter polypeptide and interfere with its activity may be used to inhibit polypeptide function. Such
10 antibodies may be generated using standard techniques, against the proteins themselves or against peptides corresponding to portions of the proteins. In some embodiments, it is preferred to use fragments of the antibody, as the smallest inhibitory fragment which binds to the target protein's binding domain. For example, peptides having an amino acid sequence corresponding to the
15 domain of the variable region of the antibody that binds to the target polypeptide may be used. Such peptides may be synthesized chemically or produced via recombinant DNA technology using methods well known in the art (e.g., see Sambrook et al., Eds., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989, or Ausubel, F. M. et al.,
20 eds. Current Protocols in Molecular Biology, 1994).

Modulation of NaCT Polypeptides:

The present invention includes methods for identifying agents that serve as substrates, modifiers, stimulators or inhibitors for one or more functional
25 activities of a NaCT polypeptide.

As used herein a "substrate" of a NaCT polypeptide is an agent that is taken up into the cells via the Na⁺-coupled citrate transporter.

As used herein a "modifier" or "modulator" of a NaCT polypeptide is an agent that alters the entry of citrate into the cell via a Na⁺-coupled citrate
30 transporter.

A modifier includes activator and stimulators of a NaCT polypeptide. As used herein an "activator" or "stimulator" of a NaCT polypeptide is an agent

that increases or enhances the entry of citrate into the cell via a Na⁺-coupled citrate transporter. “Activators” are agents that increase, open, activate, facilitate, enhance activation, agonize, or up regulate a Na⁺-coupled citrate transporter. Examples of a stimulator of a NaCT polypeptide, include, for
5 example, lithium and lithium-related salts.

A modifier includes inhibitors of a NaCT polypeptide. As used herein an “inhibitor” of a NaCT polypeptide is an agent that decreases or reduces the entry of citrate into the cell via a Na⁺-coupled citrate transporter. Inhibitors are agents that, partially or totally block activity, decrease, prevent, delay
10 activation, inactivate, or down regulate the activity or expression of a Na⁺-coupled citrate transporter. Examples of an inhibitor of a NaCT polypeptide include, for example, hydroxycitrate and other citrate analogs.

A modifier includes blockers of a NaCT polypeptide. As used herein a “blocker” of a NaCT polypeptide is an agent that binds to the NaCT polypeptide
15 and blocks the entry of citrate into the cell via the Na⁺-coupled citrate transporter but is itself not transported into the cell via the Na⁺-coupled citrate transporter.

Suitable agents can include citrate analogs, naturally occurring and synthetic ligands, antagonists, agonists, antibodies, antisense molecules,
20 ribozymes, small chemical molecules and the like. Suitable agents can also include modified versions of a NaCT polypeptide itself; for example, versions with altered activity.

Substrates, modifiers, stimulators, inhibitors, and blockers of a NaCT polypeptide may be identified using a variety of assays, including the various *in*
25 *vitro* and *in vivo* assays described herein. Assays for such agents can include, for example, expressing a NaCT polypeptide protein *in vitro*, in cells, in cell membranes, or *in vivo*, applying putative modulator compounds, and then evaluating the functional effects on activity, as described above.

Samples or assays of a NaCT polypeptide that are treated with a
30 potential activator, inhibitor, or modulator can be compared to control samples without the inhibitor, activator, or modulator to examine the extent of modification. Untreated control samples can be assigned a relative protein

activity value of 100%. Inhibition of a Na^+ -coupled citrate transporter, for example, is achieved when the activity value relative to the control is about 80%, preferably 50%, more preferably 25-0%. Activation of a Na^+ -coupled citrate transporter, for example, is achieved when the activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

An agent that modulates one or more functional activities of a NaCT polypeptide may be formulated as a composition. The compositions of the present invention may be formulated in a variety of forms adapted to the chosen route of administration. The formulations may be conveniently presented in unit dosage form and may be prepared by methods well known in the art of pharmacy. Formulations of the present invention may include, for instance, a pharmaceutically acceptable carrier. The formulations of this invention may include one or more accessory ingredients including diluents, buffers, binders, disintegrants, surface active agents, thickeners, lubricants, preservatives (including antioxidants) and the like. In addition, the formulations of this invention may further include additional therapeutic agents.

Agents of the present invention, that disrupt one or more functions of a Na^+ -coupled citrate transporter have a variety of therapeutic applications. Such agents may modify the availability of di- and tricarboxylates for cellular production of metabolic energy. Agents that disrupt the function of Na^+ -coupled citrate transporter may create a biological state similar to that of caloric restriction. Such agents may consequently lead to life span extension. Agents that disrupt the function of Na^+ -coupled citrate transporter may be useful in body weight control, the treatment of body weight disorders or the treatment of diabetes.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Functional Identity of *Drosophila melanogaster* Indy as a Cation-independent,
Electroneutral Transporter for Tricarboxylic acid-cycle Intermediates

Indy (an acronym for “I’m not dead yet”) is a gene in *Drosophila melanogaster* which, when made dysfunctional, leads to an extension of the average adult life span of the organism. Rogina et al., *Science* (2000);290: 2137–2140. In this example, the *Indy* gene-product was cloned and its functional identity established. A full-length *Indy* cDNA (SEQ ID NO:1) from a *D. melanogaster* cDNA library was isolated and the nucleotide sequence determined, as shown in Fig. 1. The cDNA codes for a protein of 572 amino acids (SEQ ID NO:2) (Fig. 1), that is called “*Drosophila* Indy” or “drIndy.” In its amino acid sequence, drIndy exhibits comparable similarity to the two known Na⁺-coupled dicarboxylate transporters in mammals; namely, NaDC1 (35% identity) and NaDC3 (34% identity). In this example, the functional characteristics of drIndy were elucidated in two different heterologous expression systems by using mammalian cells and *Xenopus laevis* oocytes. These studies showed that drIndy is a cation-independent electroneutral transporter for a variety of tricarboxylic acid-cycle intermediates, with preference for citrate compared with succinate. These characteristics of drIndy differ markedly from those of NaDC1 and NaDC3, indicating that neither of these latter transporters is the mammalian functional counterpart of drIndy. Since drIndy is a transporter for tricarboxylic acid-cycle intermediates, dysfunction of the *Indy* gene may lead to decreased production of metabolic energy in cells, analogous to caloric restriction, providing a molecular basis for the observation that disruption of the *Indy* gene function in *Drosophila* leads to extension of the average adult life span of the organism.

The protein product of the *Indy* gene is most closely related in amino acid sequence to mammalian Na⁺-coupled dicarboxylate transporters, known as NaDCs. NaDCs are secondary active transporters for dicarboxylate intermediates of the tricarboxylic acid cycle. Two different NaDCs have been

identified so far in mammalian tissues. Pajor, *Annu. Rev. Physiol* (1999);61: 663–682. These are NaDC1 and NaDC3 (a unique NaDC identified in *Xenopus laevis* is currently referred to as NaDC2). Therefore the question arises as to which one of the two NaDCs is the mammalian counterpart of *Drosophila* Indy (drIndy) in terms of biological function.

NaDC1 is Na⁺-coupled, electrogenic and exhibits a low affinity for its dicarboxylate substrates. The Michaelis–Menten constant for the prototypical substrate succinate is in the range of 0.1–4.0 millimolar (mM) (Pajor, *J. Biol. Chem.* (1995);270: 5779–5785; Pajor, *Am. J. Physiol.* (1996);270: F642–F648; and Chen et al., *J. Biol. Chem.* (1998);273: 20972–20981. This isoform is expressed primarily in the brush-border membrane of intestinal and renal epithelial cells.

NaDC3 is also Na⁺-coupled and electrogenic but exhibits relatively higher affinity for its dicarboxylate substrates compared with NaDC1. The Michaelis–Menten constant for succinate is in the range of 2–50 micromolar (μM) (Kekuda et al., *J. Biol. Chem.* (1999);274: 3422–3429; Wang et al., *Am. J. Physiol.* (2000);278: C1019–C1030; Chen et al., *J. Clin. Invest.* (1999);103: 1159–1168; and Huang et al., *J. Pharmacol. Exp. Ther.* (2000);295: 392–403). This isoform is expressed primarily in the basolateral membrane of intestinal and renal epithelial cells, sinusoidal membrane of hepatocytes, brush-border membrane of placental trophoblasts and in the plasma membrane of neurons and glial cells.

Since NaDC1 and NaDC3 differ significantly in transport characteristics and tissue-expression pattern, it is important to identify the isoform of NaDC that is the mammalian functional counterpart of drIndy. This cannot be achieved without information on the functional nature of drIndy. While Rogina et al. (*Science* (2000);290: 2137–2140) derived the amino acid sequence of a putative Indy protein on the basis of the genomic sequence and expressed sequence tags ('ESTs'), prior to the work of the present example, the full-length Indy cDNA had not been cloned nor had the transport function of Indy been established. It was not known whether drIndy is actually a Na⁺-coupled transporter for dicarboxylate anions. Therefore the present studies were

undertaken to clone the full-length drIndy cDNA and identify its transport function.

MATERIALS AND METHODS

- 5 Materials. [³H]Succinate (specific radioactivity, 40 Ci/mmol), [¹⁴C]citrate (specific radioactivity, 55 mCi/mmol), and [¹⁴C]pyruvate (specific radioactivity, 15 mCi/mmol) were purchased from Moravsek Biochemicals (Brea, CA, U.S.A.). The human retinal pigment epithelial (HRPE) cell line, used in functional expression studies, was routinely maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100units/ml penicillin and 100µg/ml streptomycin. Frogs (*Xenopus* species) were purchased from Nasco (Fort Atkinson, WI, U.S.A.).
- 10 Cloning of the drIndy cDNA. The nucleotide sequence of the putative mRNA coding for the Indy protein was first deduced from the *Drosophila* gene sequence (GenBank Accession No. AE003519; reverse complement). This sequence was used to design primers for reverse transcriptase (RT)-PCR to obtain a cDNA probe specific for Indy. The forward primer was 5'-CTCCAACTTCTTCGCTAACC-3' (SEQ ID NO:15) and the reverse primer was 5'-CTAGTGCGTCTTGTTCCTCC-3' (SEQ ID NO:16). The predicted size of the RT-PCR product was 1675 basepairs (bp). This primer pair was used to obtain a fragment of Indy cDNA by using the commercially available polyadenylated (poly(A)+) RNA from adult *D. melanogaster* (Clontech, Palo Alto, CA). This yielded a RT-PCR product of expected size. The product was subcloned in pGEM-T vector and sequenced to establish its molecular identity.
- 20 A unidirectional *Drosophila* cDNA library was then established using the commercially available poly(A)+ RNA. The SuperScript™ plasmid system (Life Technologies, Gaithersburg, MD) was employed for this purpose. The Indy-specific cDNA probe derived from RT-PCR was labeled with [α -³²P]dCTP and used to screen the *Drosophila* cDNA library under high-stringency conditions, as described in Kekuda et al., *J. Biol. Chem.* (1996);271: 18657–18661, and Prasad et al., *J. Biol. Chem.* (1998);273: 7501–7506.
- 25 DNA sequencing. Both the sense and antisense strands of the cDNA were sequenced by primer walking. Sequencing was performed by Taq
- 30

DyeDeoxy terminator cycle sequencing using an automated PerkinElmer Applied Biosystems 377 Prism DNA sequencer. The sequence was analyzed using the National Center for Biotechnology Information server, available on the world wide web at ncbi.nlm.nih.gov.

- 5 Functional expression of drIndy cDNA in mammalian cells. The functional expression of drIndy cDNA was accomplished in HRPE cells using the vaccinia virus expression system, as described in Blakely et al., *Anal. Biochem.* (1991);194: 302-308; Rajan et al., *J. Biol. Chem.* (1999);274: 29005-29010; and Kekuda et al., *J. Biol. Chem.* (1998);273: 15971-15979.
- 10 Subconfluent HRPE cells grown on 24-well plates were first infected with a recombinant (VTF7-3) vaccinia virus encoding T7 RNA polymerase and then transfected with the plasmid carrying the full-length drIndy cDNA. After 12 to 15 hours post-transfection, uptake measurements were made at 37° C with radiolabelled succinate, citrate, or pyruvate. The uptake medium was 25 mM
- 15 Hepes/Tris, pH7.5, containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5mM glucose. The time of incubation was 15 minutes, a time period representing initial-transport rates as determined from time course studies. Endogenous transport was always determined in parallel using cells transfected with pSPORT1 vector alone. The transport activity in cDNA-
- 20 transfected cells was adjusted for the endogenous activity to calculate the cDNA-specific transport activity. Experiments were performed in triplicate, and each experiment was repeated at least three times. Results are expressed as the means ± S.E.M. Since infection with vaccinia virus 'shuts off' host-cell proliferation, the cell number of HRPE cells determined immediately prior to
- 25 infection with the virus was used for calculation of transport activity.

- Functional expression of drIndy cRNA in *X. laevis* oocytes. Capped cRNA from the cloned drIndy cDNA was synthesized using the MEGAscript kit (Ambion, Austin, TX, U.S.A.). Mature oocytes from *X. laevis* were isolated by treatment with collagenase A (1.6mg/ml), manually defolliculated and
- 30 maintained at 18° C in modified Barth's medium supplemented with 10mg/l gentamicin, following procedures of Parent et al., *J. Membr. Biol.* (1992);125: 49-62. On the following day, oocytes were injected with 50 nanograms (ng) of cRNA in 50 nanoliters (nl) of water. Oocytes injected with 50 nanoliters of

water served as a control. The oocytes were used for electrophysiological studies 6 days after cRNA injection. Electrophysiological studies were performed by the conventional two-micro-electrode voltage-clamp method, following procedures described in Wang et al., *Am. J. Physiol.* (2000);278: C1019–C1030; Huang et al., *J. Pharmacol. Exp. Ther.* (2000);295: 392–403; and Kekuda et al., *J. Biol. Chem.* (1998);273: 15971–15979. Oocytes were superfused with a NaCl-containing transport buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 3 mM Hepes, 3 mM Mes and 3 mM Tris, pH7.5) followed by the same buffer containing 2mM succinate. The membrane potential was clamped at -50mV. The dependence of succinate-induced currents on Na⁺ was assessed by comparing the succinate-induced currents in the Na⁺-containing transport buffer with those in a Na⁺-free transport buffer (NaCl in the transport buffer was replaced iso-osmotically by choline chloride). Oocytes injected with human NaDC3 (hNaDC3) cRNA (Wang et al., *Am. J. Physiol.* (2000);278: C1019–C1030) were used as a positive control for succinate-induced currents.

Uptake of succinate in water-injected and cRNA-injected oocytes was measured as described previously by Fei et al., *Biochemistry* (1995);34: 8744–8751 and Nakanishi et al., *N. Am. J. Physiol* (2001);281: C1757–C1768. At 6 days after injection with water or cRNA, oocytes were incubated with [³H]succinate (7.5 µCi/ml; succinate concentration, 0.1 µM) in a NaCl-containing transport buffer at room temperature for 1 hour. After the incubation, oocytes were washed with fresh transport buffer in the absence of radiolabelled succinate four times, and then each oocyte was transferred individually into scintillation vials for determination of radioactivity.

RESULTS

Structural features of drIndy. The cloned drIndy cDNA (SEQ ID NO:1), available as GenBank Accession No. AF509505, is 2602bp long with an open reading frame (258–1976bp) coding for a protein of 572 amino acids. When compared with the amino acid sequences of hNaDC1 (Genebank Accession No. U26209) (592 amino acids) and hNaDC3 (Genebank Accession No. AF154121) (602 amino acids), there is significant similarity among the three proteins. The

sequence identity between drIndy and hNaDC1 is 35%, and that between drIndy and hNaDC3 is 34%.

Functional features of drIndy. To establish the functional identity of drIndy, the cloned cDNA was expressed heterologously in mammalian cells and assessed the ability of the clone to transport succinate. When measured in the presence of Na^+ , the uptake of succinate (40 nM) in HRPE cells transfected with drIndy cDNA was 20-fold higher than in cells transfected with vector alone (Figure 2A). This shows that drIndy indeed possesses the ability to transport the dicarboxylate succinate. However, surprisingly, drIndy was able to transport succinate not only in the presence of Na^+ , but also in the absence of Na^+ . When measured in the absence of Na^+ , the uptake of succinate in cells transfected with drIndy cDNA was still 15-fold higher than in cells transfected with vector alone. These results are in contrast with those obtained with hNaDC3 under identical conditions (Figure 2B). The uptake of succinate (40 nM), when measured in the presence of Na^+ , increased 200-fold in HRPE cells as a result of transfection with hNaDC3 cDNA. This cDNA-induced uptake was, however, completely abolished when Na^+ was omitted in the uptake medium. Similar is the case with NaDC3s from other animal species. See, Kekuda et al., *J. Biol. Chem.* (1999);274: 3422–3429 and Chen et al., *J. Clin. Invest.* (1999);103: 1159–1168. Studies by Pajor, *J. Biol. Chem.* (1995);270: 5779–5785; Pajor, *Am. J. Physiol.* (1996);270: F642–F648; and Chen et al., *J. Biol. Chem.* (1998);273: 20972–20981 have shown that the uptake of succinate mediated by NaDC1 from different animal species is also obligatorily dependent on the presence of Na^+ . Thus, although the mammalian NaDC1 and NaDC3 are Na^+ -dependent succinate transporters, drIndy is an Na^+ -independent succinate transporter.

The ability of drIndy to transport succinate remained almost the same even when Na^+ in the uptake medium was replaced with K^+ , Li^+ , *N*-methyl- D-glucamine or mannitol, suggesting that drIndy is a cation-independent succinate transporter (Table 1). Whether the drIndy-mediated transport process is dependent on an H^+ gradient was then tested by measuring the uptake of succinate at different pH values between pH 5 and 8. The uptake of succinate (40 nM) mediated by drIndy decreased gradually from 127 ± 5 to 52 ± 2

fmol/10⁶ cells per minute (means±S.E.M.) when the pH of the uptake medium was reduced from 8 to 5. These data show that drIndy is not a H⁺-coupled succinate transporter either.

5

TABLE 1

Ion-dependence of drIndy-mediated succinate transport

Uptake of succinate (40 nM) was measured in HRPE cells transfected with either vector alone or drIndy cDNA. Uptake buffer (25 mM Hepes/Tris), pH 7.5, contained one of the indicated salts (140 mM) or mannitol (280 mM).

10 In addition, all buffers contained 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5 mM glucose. Data (means±S.E.M.) are from six independent measurements. NMDG, *N*-methyl-D-glucamine.

	Succinate uptake (fmol/10 ⁶ cells per min)			
	Salt	Vector	drIndy	Fold Increase
20	NaCl	5.9±0.8	118.0±7.8	20.0
	KCl	7.0±1.2	111.0±8.5	15.9
	LiCl	5.0±0.6	122.3±4.3	24.5
	NMDG chloride	4.9±0.7	84.7±10.0	17.3
	Sodium gluconate	6.0±1.0	76.6±2.6	12.9
25	Mannitol	5.3±0.4	83.3±7.1	15.7

The substrate specificity of drIndy was then studied by assessing the ability of various monocarboxylate, dicarboxylate and tricarboxylate compounds (at a concentration of 2.5 mM) to compete with succinate (40 nM) for the transport process mediated by drIndy (Table 2). The dicarboxylate compounds 2-oxoglutarate, malate, fumarate and dimethylsuccinate were the most potent inhibitors of succinate transport mediated by drIndy. The dicarboxylate compounds maleate and malonate, and the monocarboxylate compounds lactate and β-hydroxybutyrate, were not effective. Surprisingly, the monocarboxylate and tricarboxylate compounds (pyruvate and citrate respectively) were very potent in competing with succinate for transport via

drIndy. The amino acid derivative N-acetyl aspartate was moderately effective in inhibiting succinate transport. Kinetic analysis revealed that the transport of succinate via drIndy was saturable (Figure 3). The Michaelis–Menten constant (Km) for the transport process was $40 \pm 4 \mu\text{M}$.

5

TABLE 2

Substrate specificity of drIndy

Uptake of [^3H]succinate (40 nM) was measured in HRPE cells transfected with either vector alone or drIndy cDNA. The uptake buffer was 25 mM Hepes/Tris, pH 7.5, containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5 mM glucose. Concentration of the inhibitors was 2.5 mM. Uptake in vector-transfected cells was subtracted from uptake in cDNA-transfected cells to determine drIndy cDNA-specific uptake. Data (means \pm S.E.M.) are from four independent measurements.

15

Inhibitor	cDNA-specific [^3H]succinate uptake (fmol/106 cells per min)	% Control
Control	143.0 \pm 10.8	100
20 Succinate	4.6 \pm 0.4	6
2-Oxoglutarate	3.0 \pm 1.0	5
Malate	6.8 \pm 2.7	8
Fumarate	4.8 \pm 0.3	6
Dimethylsuccinate	14.1 \pm 0.8	13
25 N-Acetylaspartate	58.4 \pm 1.5	43
Maleate	123.1 \pm 7.6	85
Malonate	98.8 \pm 7.1	71
Pyruvate	28.1 \pm 3.0	21
Lactate	111.2 \pm 3.2	77
30 β -Hydroxybutyrate	134.0 \pm 9.0	91
Citrate	18.6 \pm 3.0	13

Since the potent inhibition of drIndy-mediated, succinate transport by pyruvate and citrate was a surprise finding, the ability of drIndy to transport these two compounds was assessed directly by using radiolabelled pyruvate and citrate (Figure 4). The ability of hNaDC3 to transport these two compounds was also assessed under identical conditions, for the purposes of comparison.

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These experiments showed that drIndy possesses a marked ability to transport citrate (Figure 4A). The uptake of citrate (35 μ M) in cells transfected with drIndy cDNA was approximately 30-fold higher than in cells transfected with vector alone. The uptake of pyruvate was also stimulated in these cells as a
5 result of transfection with drIndy cDNA, but the magnitude of stimulation was comparatively much smaller. The increase in pyruvate (135 μ M) uptake as a result of transfection with drIndy cDNA was only 1.5-fold compared with transfection with vector alone, but this increase was statistically significant ($P < 0.05$). hNaDC3 differed markedly from drIndy in terms of transport of these
10 two compounds. hNaDC3 exhibited a much higher ability to transport pyruvate than to transport citrate (compare Figures 4B and 4A). These studies show a significant difference between drIndy and hNaDC3 in their relative abilities (i.e. the fold increase in transport in cDNA-transfected cells compared with vector-transfected cells) to transport pyruvate, succinate and citrate when measured
15 under identical conditions (for drIndy: citrate > succinate > pyruvate; and for hNaDC3: succinate >> pyruvate > citrate).

Since drIndy transports succinate in a cation-independent manner, whether the transport of citrate mediated by the transporter is also cation-independent was investigated. The results of these studies show that citrate
20 transport via drIndy is also cation-independent, as is the transport of succinate (Table 3). There was, however, an interesting difference between the transport of these two substrates. While the transport of succinate was not influenced by chloride, the transport of citrate was enhanced markedly when chloride was absent. Thus chloride has differential influence on the transport of succinate
25 and citrate mediated by drIndy.

TABLE 3

Ion-dependence of drIndy-mediated citrate transport

Uptake of citrate (35 μ M) was measured in HRPE cells transfected with either vector alone or drIndy cDNA. The composition of the uptake buffers was as described in Table 1. Data (means \pm S.E.M.) are from six independent measurements. NMDG, *N*-methyl D-glucamine.

Salt	Citrate uptake (pmol/10 ⁶ cells per min)		Fold Increase
	Vector	drIndy	
NaCl	2.2 \pm 0.4	57.4 \pm 3.3	26.1
KCl	1.7 \pm 0.1	33.8 \pm 2.7	19.9
LiCl	1.9 \pm 0.1	52.1 \pm 2.2	27.4
NMDG chloride	1.9 \pm 0.1	25.4 \pm 1.8	13.4
Sodium gluconate	2.2 \pm 0.1	144.2 \pm 1.6	65.6
Mannitol	1.7 \pm 0.1	26.3 \pm 0.3	15.5

The intracellular levels of various tricarboxylic acid-cycle intermediates in HRPE cells are not known. Since the influx of succinate in these cells was enhanced by drIndy, whether this influx was coupled with efflux of tricarboxylic acid-cycle intermediates from the cells was investigated. Control cells and drIndy-expressing cells were first incubated in a Na⁺-containing medium for 30 minutes in the absence or presence of 0.1mM succinate, fumarate, malate or 2-oxoglutarate. The cells were then washed, and the influx of [³H]succinate was determined. These studies showed that pre-loading of the cells with these compounds did not facilitate the influx of succinate, suggesting that drIndy-mediated succinate influx does not involve counter-transport of intracellular tricarboxylic acid-cycle intermediates.

drIndy and hNaDC3 exhibit similar affinities for succinate, the K_m values for the two transporters being 40 \pm 4 (as determined by the present study) and 20 \pm 1 μ M (as determined by Wang et al., *Am. J. Physiol.* (2000);278:C1019–C1030), respectively. The preferential ability of drIndy to transport citrate compared with hNaDC3 indicated that there may be a significant difference in the affinities of these two transporters for citrate. Therefore, the potency of citrate to inhibit the transport of succinate mediated by drIndy and

hNaDC3 was compounded under identical conditions (Figure 5). Citrate inhibited the drIndy-mediated transport of succinate with a K_i of $105 \pm 35 \mu\text{M}$. The corresponding value for hNaDC3 was $2.1 \pm 0.3 \text{ mM}$. Thus hNaDC3 exhibits a 20-fold lower affinity than drIndy for citrate.

5 It has been well established that NaDC1 and NaDC3 mediate the Na^+ -coupled transport of succinate by an electrogenic mechanism. See, for example, Pajor, *J. Biol. Chem.* (1995);270: 5779–5785; Pajor, *Am. J. Physiol.* (1996);270: F642–F648; Chen et al., *J. Biol. Chem.* (1998);273: 20972–20981; Kekuda et al., *J. Biol. Chem.* (1999);274: 3422–3429; Wang et al., *Am. J. Physiol.* (2000);278: C1019–C1030; and Chen et al., *J. Clin. Invest.* (1999);103: 1159–1168. In contrast, the transport process mediated by drIndy occurs via a Na^+ -independent mechanism. This raises the question as to whether or not the drIndy-mediated transport process is electrogenic. To address this issue, the cloned drIndy was expressed in *X. laevis* oocytes and transport function
10 assessed by measuring the uptake of radiolabelled succinate, as well as by monitoring the succinate-induced changes in membrane potential by the two-micro-electrode voltage-clamp method. Water-injected oocytes served as the control. For comparison, these experiments were also performed under identical conditions with oocytes expressing hNaDC3. The results are shown in
15 Figure 6. The uptake of radiolabelled succinate in oocytes injected with drIndy cRNA was 12-fold higher than in oocytes injected with water. This drIndy-induced uptake of succinate was, however, not influenced by the absence of Na^+ (Figure 6A). In contrast, even though the induction of radiolabelled succinate uptake by hNaDC3 in oocytes was similar to the induction caused by drIndy
20 when measured in the presence of Na^+ , the hNaDC3-induced uptake was abolished completely when Na^+ was absent in the uptake medium (Figure 6B). The changes in membrane potential were then monitored in oocytes expressing drIndy or hNaDC3 in response to succinate in the medium. Even though drIndy induced the uptake of radiolabelled succinate in oocytes, there was no
25 detectable change in membrane potential associated with the transport process (Figure 6C). This was the case irrespective of whether Na^+ was present or absent in the medium. In contrast, the presence of succinate in the medium induced marked inward currents in oocytes expressing hNaDC3, and this
30

current was obligatorily dependent on the presence of Na⁺ (Figure 6D). There was no detectable current in oocytes expressing hNaDC3 in response to succinate in the medium when Na⁺ was absent. These data show that the transport process mediated by drIndy is electroneutral.

5

DISCUSSION

The drIndy cDNA (SEQ ID NO:1) is 2602 base pairs (bp) long with a poly(A)⁺ tail. This cDNA is longer than the drIndy mRNA sequence (1872 bp long) reported by Rogina et al. (*Science* (2000);290: 2137–2140) (GenBank
10 Accession No. NM_079426), which was derived from the *Drosophila* genomic sequence (GenBank Accession No. AE003519). The additional sequence is located in the 5'-untranslated region, as well as in the 3'-untranslated region of the cloned drIndy cDNA. Comparison of the nucleotide sequence of the cloned cDNA (SEQ ID NO:1) with that of the genomic clone reveals that the *Indy* gene
15 consists of nine exons, as deduced by Rogina et al., except that the first exon is 97 bp longer than that reported in this example. Rogina et al. (*Science* (2000); 290: 2137–2140) predicted this 97 bp sequence to be a part of the first intron, but this portion of the gene is indeed expressed in mRNA, as evidenced from the sequence of the cloned cDNA (SEQ ID NO:1). The start codon is within
20 exon 2 and the stop codon is within exon 9. The drIndy mRNA reported by Rogina et al. provides sequence information only up to the stop codon. The cloned cDNA (SEQ ID NO:1) provides the sequence information on the 3'-untranslated region located in exon 9.

The mammalian proteins most similar in amino acid sequence to drIndy
25 are the Na⁺-coupled dicarboxylate ion transporters NaDC1 and NaDC3. The sequence identity between drIndy and NaDC1 or NaDC3 is 34–35%. Thus, on the basis of the primary structure alone, one cannot predict which one of these two transporters is the mammalian functional counterpart of drIndy. Therefore the functional characterization of drIndy was carried out. This was done in an
30 attempt to establish the functional identity of drIndy, and also to determine which one of the two mammalian Na⁺-coupled dicarboxylate transporters is similar to drIndy in functional characteristics. These studies have led to an

unexpected conclusion. Even though drIndy is indeed a succinate transporter, neither NaDC1 nor NaDC3 is the mammalian functional counterpart of drIndy.

There are three important functional differences between drIndy and the two mammalian Na⁺-coupled dicarboxylate transporters. The first notable
5 difference is in the ion-dependence of succinate transport mediated by the three proteins. NaDC1 and NaDC3 are strictly Na⁺-coupled succinate transporters. See, for example, Pajor, *J. Biol. Chem.* (1995);270: 5779–5785; Pajor, *Am. J. Physiol.* (1996);270: F642–F648; Chen et al., *J. Biol. Chem.* (1998);273: 20972–20981; Kekuda et al., *J. Biol. Chem.* (1999);274: 3422–3429; Wang et
10 al., *Am. J. Physiol.* (2000);278: C1019–C1030; and Chen et al., *J. Clin. Invest.* (1999);103: 1159–1168. In the absence of Na⁺, the mammalian transporters do not exhibit any detectable ability to transport succinate. In contrast, Na⁺ is not essential for the transport of succinate via drIndy. The ability of drIndy to mediate the transport of succinate remains the same even when Na⁺ in the
15 medium is replaced iso-osmotically by other univalent inorganic cations, such as K⁺ or Li⁺, or by the non-ionizable organic solute mannitol. The second notable difference is in substrate selectivity. drIndy transports the tricarboxylate citrate much more efficiently than the dicarboxylate succinate.

This is not the case with NaDC1 and NaDC3. These two mammalian
20 proteins transport succinate much more efficiently than citrate. With respect to pyruvate, drIndy possesses a small, but detectable, ability to transport this monocarboxylate. Surprisingly, NaDC3 shows a much higher ability to transport pyruvate. The third important difference is in the electrogenic nature of these transporters. NaDC1 and NaDC3 are electrogenic transporters for
25 which the transport function is associated with a net transfer of positive charge into the cells. In contrast, drIndy is electroneutral. The transport function of drIndy is not associated with membrane depolarization, as evidenced from the absence of substrate- induced inward currents in oocytes functionally expressing drIndy. Thus, whereas the mammalian NaDCs are Na⁺-coupled
30 electrogenic transporters with preference towards dicarboxylate groups, drIndy is a cation-independent electroneutral transporter with preference for the tricarboxylate groups of citrate.

Example 2

Structure, Function, and Expression Pattern of a Novel Sodium-coupled Citrate Transporter (NaCT) Cloned from Mammalian Brain

5 Citrate plays a pivotal role not only in the generation of metabolic energy but also in the synthesis of fatty acids, isoprenoids, and cholesterol in mammalian cells. Plasma levels of citrate are the highest (approximately 135 μM) among the intermediates of the tricarboxylic acid cycle. This example reports on the cloning and functional characterization of a plasma membrane
10 transporter (NaCT for Na^+ -coupled citrate transporter) from rat brain that mediates uphill cellular uptake of citrate coupled to an electrochemical Na^+ gradient. NaCT consists of 572 amino acids and exhibits structural similarity to the members of the Na^+ -dicarboxylate cotransporter/ Na^+ -sulfate cotransporter (NaDC/NaSi) gene family including the recently identified *Drosophila* Indy. In
15 rat, the expression of NaCT is restricted to liver, testis, and brain. When expressed heterologously in mammalian cells, rat NaCT mediates the transport of citrate with high affinity (Michaelis-Menten constant, approximately 20 μM) and with a Na^+ :citrate stoichiometry of 4:1. The transporter does interact with other dicarboxylates and tricarboxylates but with considerably lower affinity.
20 In mouse brain, the expression of NaCT mRNA is evident in the cerebral cortex, cerebellum, hippocampus, and olfactory bulb. NaCT represents the first transporter to be identified in mammalian cells that shows preference for citrate over dicarboxylates. This transporter is likely to play an important role in the cellular utilization of citrate in blood for the synthesis of fatty acids and
25 cholesterol (liver) and for the generation of energy (liver and brain). NaCT thus constitutes a potential therapeutic target for the control of body weight, cholesterol levels, and energy homeostasis.

The cloning of *Drosophila* Indy, presented in Example 1, produced unexpected results. *Drosophila* Indy does possess the ability to transport
30 succinate as do mammalian NaDCs but the transport is Na^+ -independent. Furthermore, unlike mammalian NaDCs, *Drosophila* Indy transports citrate very effectively. The affinity for citrate is several fold greater in the case of *Drosophila* Indy than in the case of mammalian NaDCs. These findings

suggested that neither NaDC1 nor NaDC3 is the mammalian ortholog of *Drosophila* Indy. Therefore, the Genbank database was searched to see if there are additional transporters in mammals that are structurally related to NaDC1 and NaDC3. This search led to the identification of a novel mammalian transporter that is structurally similar to mammalian NaDCs as well as to *Drosophila* Indy. Interestingly, this new transporter transports citrate much more effectively than succinate, a characteristic more similar to that of *Drosophila* Indy than to that of mammalian NaDCs. However, the transport process is coupled to Na⁺, a feature distinct from that of *Drosophila* Indy but similar to that of mammalian NaDCs. Based on these functional characteristics, this novel transporter has been designated NaCT (for Na⁺-coupled citrate transporter). This represents the first sodium-coupled transporter in mammals to be identified that shows preference for citrate as a substrate.

EXPERIMENTAL PROCEDURES

Materials. [¹⁴C]Citrate (specific radioactivity, 55 mCi/mmol), [³H]succinate (specific radioactivity, 40 Ci/mmol), and [¹⁴C]pyruvate (specific radioactivity, 15 mCi/mmol) were purchased from Moravsek Biochemicals (Brea, CA). The human retinal pigment epithelial (HRPE) cell line was maintained in Dulbecco's minimum essential medium/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Lipofectin was purchased from Invitrogen. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Magna nylon transfer membranes used in library screening were purchased from Micron Separations (Westboro, MA). Unlabeled monocarboxylates, dicarboxylates, and tricarboxylates were obtained from Sigma.

Cloning of NaCT from Rat Brain. A search of the Genbank database for murine established sequence tags (ESTs) with the amino acid sequence of *Drosophila* Indy as a query identified several ESTs whose predicted amino acid sequences showed significant similarity to that of mammalian sodium-coupled dicarboxylate transporters NaDC1 and NaDC3. Many of these ESTs were identical to murine NaDC1 and NaDC3, which have been already cloned and functionally characterized. Pajor et al., *Am. J. Physiol.* (2001);280: C1215-

C1223; and Pajor and Sun, *Am. J. Physiol.* (2000);279: F482-F490. However, there were three ESTs (Genbank Accession Nos. BB261903, BB393630, and BB641100) with overlapping nucleotide sequences, and the predicted amino acid sequence from these ESTs did not correspond to that of murine NaDC1 or NaDC3. However, there was significant similarity between this sequence and that of NaDC1, NaDC3, and *Drosophila* Indy. There is no information in the literature on the functional identity of this new putative mammalian transporter. The sequence similarity however suggested that it might represent a new member of the sodium/dicarboxylate cotransporter gene family. The overlapping nucleotide sequences of two of these ESTs were used to design primers for RT-PCR to obtain a cDNA probe that is specific for this transporter. The forward primer was 5'-TCTTTTCTCCCTCCAGTCAGT-3' (SEQ ID NO:17) and the reverse primer was 5'-GGCAATCTTCTCGGTGTC-3' (SEQ ID NO:18). The predicted size of the RT-PCR product, based on the positions of the primers, was 943 bp. Since both of these ESTs were identified from a mouse cerebral cortex cDNA library, poly(A) RNA from mouse brain was used as the template for RT-PCR to obtain the probe. This yielded a cDNA product of expected size. The product was subcloned in pGEM-T vector and sequenced to establish its molecular identity. The cDNA was labeled with [α -³²P]dCTP by random priming using the ready-to-go oligolabeling beads (Amersham Biosciences). This probe was used to screen a rat brain cDNA library under medium stringency conditions. The screening of the library was as described by Helfand and Rogina (*Cell Differ.* (2000);29: 67-80) and Kekuda et al. (*J. Biol. Chem.* (1998);273: 15971-15979). Positive clones were purified by secondary or tertiary screening. The library used here was originally established in pSPORT1 vector at SalI/NotI site using RNA isolated from rat total brain (Rajan et al., *J. Biol. Chem.* (1999);274: 29005-29010; Seth et al., *J. Neurochem.* (1998);70: 922-931; Wang et al., *Am. J. Physiol.* (1998); 275: C967-C975; and Huang et al., *J. Pharmacol. Exp. Ther.* (2000);295: 392-403). The cDNA inserts in the vector were under the control of T7 promoter.

DNA Sequencing. Both sense and antisense strands of the cDNA were sequenced by primer walking. Sequencing was performed by Taq DyeDeoxy terminator cycle sequencing using an automated PE Applied Biosystems 377

Prism DNA sequencer. The sequence was analyzed using the National Center for Biotechnology Information server on the world wide web at ncbi.nlm.nih.gov.

5 Northern Analysis. The expression pattern of NaCT mRNA in rat tissues was analyzed by Northern hybridization using a commercially available rat multiple tissue blot. The blot was hybridized with a ^{32}P -labeled rat NaCT-specific cDNA probe under high stringency conditions. The same blot was then hybridized with a cDNA probe specific for rat -actin as an internal control to detect the presence of RNA in each lane.

10 Functional Expression in HRPE Cells. The cloned transporter was expressed heterologously in HRPE cells using the vaccinia virus expression technique as described by Kekuda et al., *J. Biol. Chem.* (1998);273: 15971-15979; Rajan et al., *J. Biol. Chem.* (1999);274: 29005-29010; Seth et al., *J. Neurochem.* (1998);70: 922-931; Wang et al., *Am. J. Physiol.* (1998); 275: 15 C967-C975; Huang et al., *J. Pharmacol. Exp. Ther.* (2000);295: 392-403; and Nakanishi et al., *Am. J. Physiol.* (2001);281: C1757-C1768). This is a transient expression system. Functional analysis of the cloned transporter was carried out with this experimental system. Briefly, subconfluent HRPE cells grown on 24-well plates were first infected with a recombinant (VTF7-3) vaccinia virus
20 encoding T7 RNA polymerase and then transfected with the plasmid carrying the full-length rat NaCT cDNA. For comparison with the newly cloned transporter, rat NaDC3 cDNA isolated from a placental cDNA library (Kekuda et al., *J. Biol. Chem.* (1999);274: 3422-3429) was used in some of the functional studies. After 12-15 hours post-transfection, uptake measurements were made
25 at 37° C with radiolabeled citrate, succinate, or pyruvate. The uptake medium in most experiments was 25 mM Hepes/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 5 mM glucose. The time of incubation was 15 minutes for citrate and 1 minute for succinate. These time periods were chosen from time course studies with respective substrates to
30 represent linear uptake conditions. Endogenous uptake was always determined in parallel using the cells transfected with pSPORT1 vector alone. The uptake activity in cDNA-transfected cells was adjusted for the endogenous uptake activity to calculate the cDNA-specific activity. In experiments in which the

cation and anion dependence of the uptake process was investigated, NaCl was replaced isoosmotically with LiCl, KCl, N-methyl-D-glucamine (NMDG) chloride, sodium gluconate, or mannitol. When the influence of pH on the uptake process was investigated, uptake buffers of different pH values were prepared by appropriately altering the concentrations of Tris, Mes, and Hepes. Saturation kinetics was evaluated by non-linear regression analysis, and the kinetic parameters derived from this method were confirmed by linear regression analysis. The Na^+ :citrate stoichiometry was calculated by measuring citrate uptake with varying concentrations of Na^+ and then by analyzing the data according to Hill equation. The concentrations of Na^+ were varied by appropriately changing the concentrations of NaCl and NMDG chloride without changing the osmolality. The influence of membrane potential on the uptake process was investigated by measuring the uptake in the presence of high concentrations of K^+ , a condition, which leads to depolarization of the membrane. Experiments were done in duplicate or triplicate, and each experiment was repeated two or three times. Results are expressed as means \pm standard error (S.E.).

In Situ Hybridization. The expression pattern of NaCT mRNA in mouse brain was investigated by *in situ* hybridization as previously described in Huang et al., *J. Pharmacol. Exp. Ther.* (2000);295: 392-403; Wu et al., *J. Biol. Chem.* (1998);273: 32776-32786; Wu et al., *J. Pharmacol. Exp. Ther.* (1999);290: 1482-1492; Wu et al., *Biochim. Biophys. Acta* (2000);1466: 315-327; Seth et al., *Biochim. Biophys. Acta* (2001);1540: 59-67; Bridges et al., *J. Biol. Chem.* (2000);275: 20676-20684; and Ola et al., *Brain Res. Mol. Brain Res.* (2001);95: 86-95. For the preparation of the mouse NaCT-specific riboprobe, as the template a 363 bp cDNA derived by PCR from the approximately 0.9 kilobasepair (kbp) RT-PCR product that was generated with mouse brain RNA was used. This cDNA was subcloned in pGEM-T vector, and T7 RNA polymerase and SP6 RNA polymerase were used to prepare the sense and antisense riboprobes after linearization of the plasmid with appropriate restriction enzymes. The riboprobes were labeled using a digoxigenin-labeling kit (Roche Molecular Biochemicals).

Whole brains from mice were frozen immediately in Tissue-Tek OCT, sectioned at 10- μ m thickness, and fixed in 4% paraformaldehyde. Sections were rinsed in ice-cold phosphate-buffered saline and treated with 1% diethylpyrocarbonate. Permeabilization of the sections was carried out with proteinase K (1 μ g/ml) in phosphate-buffered saline for 4 minutes. Proteinase K activity was terminated by rinsing the sections with glycine (2 mg/ml) in phosphate-buffered saline. Sections were then washed in phosphate-buffered saline, equilibrated in 5 \times SSC (100 mM NaCl/15 mM sodium citrate), and prehybridized for 2 hours at 58 $^{\circ}$ C in 50% (v/v) formamide, 5 \times SSC, 2% (w/v) blocking agent (provided with the digoxigenin nucleic acid detection kit), 0.1% (w/v) N-laurylsarcosine, and 0.02% (w/v) sodium dodecyl sulfate. Sections were hybridized with digoxigenin-UTP-labeled antisense or sense (negative control) riboprobes (1 μ g/ml) and were incubated overnight at 58 $^{\circ}$ C. Post-hybridization washings were done twice in 2 \times SSC at 58 $^{\circ}$ C, twice in 1 \times SSC at 55 $^{\circ}$ C, and twice in 0.1 \times SSC at 37 $^{\circ}$ C. Hybridization signals were then detected immunologically by using an antibody specific for digoxigenin. This was done by first washing the sections in a buffer containing 0.1 M maleic acid and 0.15 M NaCl (pH 7.5) and then exposing the washed sections to 1% blocking agent in the same buffer. Sections were then incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (1 in 5000 dilution) overnight at 4 $^{\circ}$ C. Following this, sections were washed in the preceding wash buffer containing levamisole (0.2 mg/ml) twice for 10 minutes and equilibrated with 100 mM Tris/HCl buffer (pH 9.5) containing 100 mM NaCl and 50 mM MgCl₂. The color reaction was developed in NBT/BCIP (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate). Slides were washed in distilled water and coverslipped but not counterstained so that the purplish-red colored precipitate, indicative of a positive reaction, could be visualized in the sections.

30 RESULTS

Structural Features of Rat NaCT. As shown in Figure 7, the cloned rat NaCT cDNA (SEQ ID NO:3) is 3254 bp long with a poly(A) tail and an open reading frame (24-1742 bp) coding for a protein of 572 amino acids (SEQ ID

NO:4). The nucleotide sequence of rat NaCT cDNA is also available as Genbank Accession No. AF522186. This rat transporter is new and has not been reported previously in the literature. Based on the amino acid sequence, rat NaCT belongs to the NaDC/NaSi (sodium-dicarboxylate
5 cotransporter/sodium-sulfate cotransporter) gene family which, to date, consists of three sodium-coupled dicarboxylate transporters (NaDC1, NaDC2, and NaDC3) and two sodium-coupled sulfate transporters (NaSi and SUT1) (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682). However, the rat NaCT is structurally more closely related to sodium-coupled dicarboxylate transporters than to
10 sodium-coupled sulfate transporters. It shows a 44-50% sequence identity with rat NaDCs (Fig. 8) (Inoue et al., *J. Biol. Chem.* (2002);277: 39469-39476).

It also shows significant structural similarity to *Drosophila* Indy (34% identity). Hydropathy analysis suggests that rat NaCT possesses eleven putative transmembrane domains, similar to the previously known sodium-coupled
15 dicarboxylate transporters. A search of the Genbank database using the rat NaCT amino acid sequence as a query has revealed that a putative human ortholog of rat NaCT is located on chromosome 17 upstream of the gene coding for NaDC1 (17p13 in the case of the gene coding for NaCT and 17p11.1-q11.1 in the case of the gene coding for NaDC1) (Pajor, *Am. J. Physiol.* (1996);270:
20 F642-F648).

Tissue Expression Pattern of NaCT mRNA in the Rat. Northern blot analysis shows that NaCT mRNA (approximately 3.2 kb) is expressed in a restricted manner in rat tissues. The expression is evident only in the liver, testis, and brain (Fig. 9). The level of expression in the liver and testis is much
25 higher than in the brain. There is only a single transcript detectable.

Functional Features of Rat NaCT. The transport function of rat NaCT was assessed in a heterologous expression system in a mammalian cell line (HRPE) using the vaccinia virus expression technique. Since rat NaCT shows high structural similarity to the sodium-coupled dicarboxylate transporters, the
30 ability of rat NaCT to transport succinate in the presence of a Na⁺ gradient was first tested (Fig. 10A). The uptake of succinate (80 nM) in cells transfected with rat NaCT cDNA was about 7-fold higher than in cells transfected with vector alone, indicating that the cloned transporter does possess the ability to

mediate the uptake of this dicarboxylate. It was then tested whether pyruvate (a monocarboxylate) and citrate (a tricarboxylate) are recognized as transport substrates by rat NaCT. Surprisingly, the uptake of both of these compounds (pyruvate, 135 μ M; citrate, 35 μ M) was higher in cDNA-transfected cells than in vector-transfected cells. The cDNA-induced increase in pyruvate uptake was marginal (approximately 3-fold) whereas the increase was much more marked in the case of citrate (approximately 90-fold). This was very interesting because the previously described NaDCs possess much lower ability to transport citrate than to transport succinate (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682). Since rat NaCT was able to transport citrate preferentially, this tricarboxylate was used as a substrate for subsequent functional characterization of the transporter. The cDNA-mediated uptake of citrate was linear even up to 30 minutes (Fig. 10B). The uptake process operated maximally at pH 7 (Fig. 10C).

The involvement of Na⁺ in the uptake process mediated by rat NaCT was evaluated by monitoring the uptake of citrate in vector-transfected cells and in rat NaCT cDNA-transfected cells in the presence and absence of Na. This was done by isoosmotically replacing NaCl in the uptake medium with NMDG chloride, KCl, LiCl, or mannitol (Table 4). The cDNA-specific uptake was almost completely abolished when Na⁺ was removed from the medium. The uptake process was however not dependent on Cl because the replacement of Cl with gluconate had only a small effect on the cDNA-specific citrate uptake.

The substrate specificity of rat NaCT was examined by competition studies in which the ability of various unlabeled compounds (2.5 mM) to compete with [¹⁴C]citrate (7 μ M) for uptake via rat NaCT (Table 5) was assessed. Unlabeled citrate was the most potent inhibitor of [¹⁴C]citrate uptake mediated by rat NaCT. Among the dicarboxylates, succinate and malate were very potent inhibitors. Fumarate and α -ketoglutarate were comparatively less effective. Maleate, the cis isomer of fumarate, was one of the least potent inhibitors, showing stereoselectivity of the transporter. The monocarboxylates pyruvate and lactate were not effective in inhibiting [¹⁴C]citrate uptake.

TABLE 4

Ion dependence of citrate uptake via rat NaCT

Uptake of [14 C]citrate (7 μ M) was measured in vector-transfected HRPE cells and in rat NaCT cDNA-transfected HRPE cells at pH 7.5 in the presence of various inorganic salts or mannitol. Values represent means \pm S.E.

Citrate Uptake				
Salt	Vector	cDNA	cDNA-specific	%
		<i>pmol/10⁶ cells/min</i>		
10 NaCl	0.84 \pm 0.14	38.8 \pm 3.3	38.0 \pm 3.3	100
NMDG chloride	1.22 \pm 0.22	1.6 \pm 0.3	0.4 \pm 0.3	1
KCl	0.49 \pm 0.03	0.6 \pm 0.1	0.1 \pm 0.1	0
LiCl	0.51 \pm 0.02	1.6 \pm 0.1	1.1 \pm 0.1	3
Sodium gluconate	0.48 \pm 0.03	29.6 \pm 2.3	29.1 \pm 2.3	77
15 Mannitol	0.59 \pm 0.03	0.7 \pm 0.1	0.1 \pm 0.1	0

Kinetic Features of Rat NaCT. Citrate uptake mediated by rat NaCT was saturable with a K_t of $18 \pm 4 \mu\text{M}$ (Fig. 11A). Kinetic analysis of Na^+ dependence of citrate uptake via rat NaCT showed that the relationship between the uptake and Na^+ concentration was sigmoidal, indicating the involvement of multiple Na^+ ions per transport cycle (Fig. 11B). The data were analyzed by Hill equation to determine the Na^+ :citrate stoichiometry. This analysis gave a value between 3 and 4 (3.3 ± 0.3) for the Hill coefficient (nH). Since citrate exists predominantly as a trivalent anion under the experimental conditions (i.e. pH 7.5), cotransport of citrate with 3 Na^+ ions will render the transport process electrically silent whereas cotransport with 4 Na^+ ions will render the process electrogenic. Therefore, whether or not the rat NaCT-mediated citrate uptake in the presence of Na^+ is influenced by membrane potential was investigated. For this purpose, the cDNA-specific citrate uptake between control conditions and membrane depolarizing conditions (i.e. high extracellular K^+) (Fig. 11C) was compared. The uptake was inhibited significantly ($43 \pm 2\%$) when membrane was depolarized, indicating that the uptake process is influenced by membrane potential. Since depolarization inhibits the uptake, one can conclude that the uptake process mediated by rat NaCT is electrogenic associated with a net transfer of positive charge into the cells. This would then suggest that at least 4 Na^+ are cotransported with one citrate in the transport process.

TABLE 5

Substrate specificity of rat NaCT

Uptake of [^{14}C]citrate (14 μM) was measured in vector-transfected HRPE cells and in rat NaCT cDNA-transfected HRPE cells in the absence or presence of various monocarboxylates, dicarboxylates, or tricarboxylates (2.5 mM). Data (means \pm S.E.) represent only cDNA-specific uptake.

Unlabeled compound		[^{14}C]Citrate uptake	
		<i>Pmol/10⁶ cells/min</i>	%
10	None	108.3 \pm 5.5	100
	Citrate	1.5 \pm 0.1	1
	Succinate	5.7 \pm 0.2	5
	Malate	7.8 \pm 0.1	7
15	Fumarate	17.7 \pm 1.4	16
	α -Ketoglutarate	36.5 \pm 2.1	34
	Maleate	78.8 \pm 1.5	73
	Pyruvate	89.4 \pm 7.2	83
20	Lactate	89.6 \pm 2.0	83

The affinities of rat NaCT for different tricarboxylates (citrate, isocitrate, and cis-aconitate) and dicarboxylates (succinate, fumarate, and α -ketoglutarate) was then compared by monitoring the potencies of these compounds to inhibit the uptake of [^{14}C]citrate (14 μM) mediated by rat NaCT (Fig. 12A). Among these compounds, citrate was the most potent inhibitor of rat NaCT-mediated [^{14}C]citrate uptake. The inhibitory potencies of other compounds were in the following order: succinate > fumarate = cis-aconitate > α -ketoglutarate. While citrate was the most potent inhibitor, isocitrate had no detectable inhibitory effect. The IC_{50} values (i.e. concentrations of inhibitors causing 50% inhibition) calculated for citrate and succinate, the two most potent inhibitors, from these dose-response curves are 28 ± 4 and 172 ± 24 μM , respectively. The IC_{50} for other compounds are several fold higher than these values. Using the IC_{50} values for citrate and succinate, the corresponding inhibition constants was calculated by the method of Cheng and Prusoff (Cheng et al., *Biochem. Pharmacol.* (1973);22: 3099-3108). The inhibition constants

for these two compounds are 16 ± 2 and 98 ± 14 μM , respectively. These data show that, among the compounds tested, rat NaCT possesses the highest affinity for citrate. Thus, the affinity of rat NaCT for succinate is 6-fold lower than that for citrate. The affinities for other dicarboxylates and tricarboxylates are even
5 lower. Thus, citrate is relatively a specific substrate for rat NaCT.

For comparison, the relative affinity of rat NaDC3 for these compounds was analyzed. This was done in a similar way by assessing the potencies of these compounds to inhibit the uptake of [^3H]succinate (80 nM) mediated by rat NaDC3 cloned from placenta using the same heterologous expression system
10 (Fig. 12B). The results show that rat NaDC3 interacts with dicarboxylates (succinate, fumarate, and α -ketoglutarate) much more preferentially than with tricarboxylates (citrate, isocitrate, and cis-aconitate). The inhibition constants for the three dicarboxylates are in the range of 15-60 μM whereas the corresponding values for the three tricarboxylates are in the range of 2-4 mM.
15 Thus, rat NaDC3 recognizes a number of dicarboxylates as preferential substrates and has very low affinity for tricarboxylates. This qualifies rat NaDC3 to be recognized as a sodium-coupled dicarboxylate transporter. On the contrary, rat NaCT transports citrate but interacts with other tricarboxylates very poorly. It has to be noted that this transporter does recognize succinate as
20 a substrate, but its affinity for succinate is several fold lower than for citrate.

Furthermore, other dicarboxylates interact with the transporter very poorly. Based on these data, the newly cloned transporter was identified as a sodium-coupled citrate transporter.

Next the physiological relevance of the differential substrate specificity
25 of rat NaCT and rat NaDC3 was assessed. Plasma contains significant levels of citrate (approximately 25 mg/liter) and succinate (approximately 5 mg/liter). α -ketoglutarate, oxaloacetate, fumarate, and malate are also present in the plasma but at much lower levels. Normal fasting levels of citrate and succinate in the plasma are approximately 135 and approximately 40 μM , respectively. The
30 plasma levels of citrate increase by 25-30% above the fasting levels after meal. To investigate the physiological relevance of the substrate specificity of NaCT and NaDC3, the ability of these two transporters to mediate the transport of citrate and succinate in the presence of a Na^+ gradient when the medium

contained physiological concentrations of these two compounds (i.e., 135 μ M citrate and 40 μ M succinate) was assessed. Under these conditions, the values for succinate uptake mediated by rat NaCT and rat NaDC3 were 0.005 ± 0.001 and 0.48 ± 0.01 nmol/ 10^6 cells/minute, respectively. In contrast, the values for citrate uptake mediated by these two transporters were 0.24 ± 0.01 and 0.04 ± 0.01 nmol/ 10^6 cells/minute, respectively. These data show that, at physiological concentrations of succinate and citrate found in plasma, NaDC3 functions primarily to transport succinate whereas NaCT functions primarily to transport citrate.

10 Analysis of Expression Pattern of NaCT mRNA in Mouse Brain by *In Situ* Hybridization. To determine the expression pattern of NaCT mRNA in mouse brain, sagittal sections of mouse brain were subjected to *in situ* hybridization with digoxigenin-labeled riboprobes specific for mouse NaCT. Hybridization with antisense probe has revealed that the NaCT mRNA is
15 expressed widely in the mouse brain, primarily in the neurons of the cerebral cortex, hippocampal formation, cerebellum, and olfactory bulb (Fig. 13A). The signals are specific as evidenced from markedly reduced signals with sense probe (Fig. 13B). In the cerebellum, the hybridization signals with antisense probe are most intense in the Purkinje cell bodies, followed by the neurons in
20 the granular layer (Fig. 13C and 13D). Expression is also evident in the interneurons of the molecular layer, namely the stellate cells located in the outer portion of the molecular layer and the basket cells located close to the border between the molecular and Purkinje layers. While the white matter is negative for hybridization signal, the deep cerebellar nuclei are intensely positive for
25 expression. In the hippocampal formation (Fig. 13E and 13F), the pyramidal cells in the cornus ammonis regions CA3, CA2, and CA1 are highly positive for mRNA expression, followed by neurons in the subiculum. Granule cells, which are the projection neurons of the dentate gyrus, are intensely positive for the hybridization signal. Similarly, the interneurons of the polymorphic layer in the
30 dentate gyrus are also positive for expression. The molecular layer that has very few cell bodies is largely negative. In the cerebral cortex, the expression is widespread. Based on the expression pattern in the hippocampal formation and

cerebellum, the observed expression in the cerebral cortex is likely to be restricted primarily to neurons.

DISCUSSION

5 Functional Differences between NaCT and NaDC1/NaDC3. NaCT represents the newest member of the NaDC/NaSi gene family. Structurally and functionally, NaCT is closely related to NaDC1 and NaDC3. But, there are significant differences among these three transporters in terms of substrate specificity, substrate affinity, and tissue expression pattern. NaCT is Na⁺-
10 coupled and exhibits much higher affinity for citrate than for succinate. This is in contrast to NaDC1 and NaDC3 that, though Na⁺-coupled as NaCT, exhibit much higher affinity for succinate than for citrate. It has to be pointed out here that NaDC1 and NaDC3 do transport citrate, but only the divalent form of citrate is recognized as a substrate by these two transporters (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682).

 In contrast, it is the trivalent form of citrate that serves as the substrate for NaCT. This conclusion is supported by the differential influence of pH on citrate uptake via NaCT, NaDC1, and NaDC3. Even though a change of pH from 8.5 to 7.0 enhances citrate uptake via NaCT, further decrease in pH
20 actually interferes with uptake. Citrate (pK₁, 3.1, pK₂, 4.8, and pK₃, 6.4) exists predominantly (approximately 75%) as a trivalent anion at pH 7.0 and NaCT-mediated uptake of citrate is maximal under these conditions. The fraction of the divalent form of citrate becomes significantly greater when the pH is made more acidic than pH 7.0. If the divalent form of citrate is the preferred substrate
25 for NaCT, the uptake is expected to increase at pH more acidic than 7.0. Instead, the uptake decreases markedly when the pH is made more acidic. This is in contrast to NaDC3, which transports citrate at a much higher rate at pH 6.0 than at pH 7.5 (Wang et al., *Am. J. Physiol.* (2001);278: C1019-C1030). Similar results have been obtained with NaDC1 (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682). Additional evidence for the transport of citrate via NaCT
30 as a trivalent anion stems from the Na⁺:citrate stoichiometry. The Hill coefficient is greater than 3 and the uptake process at pH 7.5 is electrogenic. Since citrate exists 90% as a trivalent anion at pH 7.5, the electrogenic nature of

the uptake process suggests that the number of Na⁺ ions involved in the process is greater than 3. In the case of NaDC1 and NaDC3, the number of Na⁺ ions involved in the uptake process is 3 and the process is still electrogenic, suggesting that these transporters prefer to interact with the divalent anionic forms of their substrates.

Another notable difference between NaCT and the two isoforms of NaDC is in substrate selectivity. NaCT is relatively very specific for citrate. Even though it recognizes the tricarboxylate citrate as its substrate, it interacts very poorly with other structurally related tricarboxylates such as isocitrate and cis-aconitate. NaCT does transport succinate, but its affinity for this dicarboxylate is 6-fold less than for citrate. In contrast, NaDC3 exhibits comparable affinity for several structurally related dicarboxylates such as succinate, malate, fumarate, oxaloacetate, and α -ketoglutarate. Furthermore, NaDC3 does not differentiate among the tricarboxylates citrate, isocitrate, and cis-aconitate though the affinity of the transporter for these tricarboxylates is several fold lower than for the dicarboxylates. This indicates that NaDC3 has broad substrate selectivity among the dicarboxylates and the divalent forms of tricarboxylates. The same is true with NaDC1 (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682). This is not the case with NaCT. The substrate selectivity of NaCT is comparatively more restricted. This is particularly evident from the lack of interaction between NaCT and isocitrate. Citrate differs from isocitrate only in the position of the hydroxyl group. Thus, among the tricarboxylates tested, citrate has the highest affinity for NaCT whereas isocitrate has no detectable affinity for the transporter. These data indicate that NaCT is primarily a citrate transporter. This is especially appreciable when the abilities of NaCT and NaDC3 to transport succinate or citrate are compared under physiological conditions with plasma concentration of citrate far exceeding the combined concentrations of other potential substrates. Under these conditions, NaCT transports primarily citrate whereas NaDC3 transports primarily succinate. Therefore, NaCT does not possess broad specificity toward structurally related tricarboxylates. Its substrate selectivity is essentially restricted to citrate. On the other hand, NaDC1 and NaDC3 are Na⁺-coupled transporters with broad specificity toward structurally related dicarboxylates.

Differences in Tissue Expression Pattern Between NaCT and NaDC1/NaDC3. In the rat, NaCT mRNA is expressed primarily in the liver, testis, and brain. In contrast, NaDC1 mRNA is expressed mostly in the small intestine and kidney whereas NaDC3 mRNA is expressed primarily in the kidney, small intestine, liver, placenta, and brain (Chen et al., *J. Biol. Chem.* (1998);273: 20972-20981, and Kekuda et al., *J. Biol. Chem.* (1999);274: 3422-3429). Thus, these three transporters differ significantly in the expression pattern in rat tissues. Even though NaCT mRNA and NaDC3 mRNA are expressed in rodent brain, the distribution pattern is very different. NaCT mRNA is detectable very widely in the brain. The expression is most abundant in the hippocampal formation, cerebellum, cerebral cortex, and olfactory bulb. On the other hand, NaDC3 mRNA is expressed primarily in the meningeal layers of supporting tissue that surround the brain. Its expression is evident but weak in the cerebral cortex, hippocampus, and cerebellum. Furthermore, NaCT mRNA expression is found mostly in neurons whereas NaDC3 mRNA expression occurs mostly in glial cells.

Physiologic and Therapeutic Significance of NaCT as a Citrate Transporter. NaCT is the first plasma membrane transporter described in mammals which functions primarily in the cellular uptake of citrate. The plasma concentration of citrate is 135 μ M. Since the K_m for the transport of citrate via NaCT is approximately 20 μ M, the transporter is likely to play an efficient role in the cellular entry of citrate under physiological conditions. Citrate occupies a pivotal position in cellular metabolism. It is not only an intermediate in the citric acid cycle that is the primary site of biological energy production in most cells, but also is a source of cytosolic acetyl CoA for the synthesis of fatty acids, isoprenoids, and cholesterol and for the elongation of fatty acids. Acetyl CoA present in the cytoplasm originates from citrate produced within mitochondria. A tricarboxylate transporter located in the inner mitochondrial membrane mediates the electroneutral efflux of citrate from the mitochondrial matrix in exchange for cytosolic malate or succinate. Following the entry into the cytoplasm, citrate is cleaved by ATP-citrate lyase to generate acetyl CoA.

The identification of NaCT as a plasma membrane citrate transporter indicates that citrate in the circulation may serve as an important source of cytoplasmic citrate. NaCT is a highly concentrative transporter with a $\text{Na}^+:\text{citrate}$ stoichiometry of 4:1. It is electrogenic and thus the cellular entry of citrate via NaCT is energized not only by a Na^+ gradient but also by the membrane potential. Citrate that enters the cells via NaCT may either serve as a precursor for the biosynthesis of fatty acids and cholesterol or enter the mitochondrial matrix to serve as an intermediate in the citric acid cycle. The choice between these two pathways will depend on the hormonal milieu and metabolic state of the cell. The expression of NaCT in the liver is of physiologic importance in this respect because this organ plays a vital role in the synthesis of fatty acids, isoprenoids, and cholesterol. Therefore, NaCT will be an important therapeutic target for controlling hepatic production of fatty acids and cholesterol, with a selective blocker or inhibitor of this transporter preventing hepatic utilization of citrate in blood in these biosynthetic processes. Since NaCT is expressed in the brain mostly in neurons, this transporter will play an important role in supplying citrate for these cells as a metabolic precursor for production of ATP via citric acid cycle.

Interestingly, even though the mitochondrial tricarboxylate transporter (Palmieri, *FEBS Lett.* (1994);346: 48-54; Kaplan et al., *J. Biol. Chem.* (1993);268: 13682-13690) and the plasma membrane NaCT transport citrate, there is no structural similarity between these two transporters. The mitochondrial tricarboxylate transporter consists of 298 amino acids and possesses six putative transmembrane domains (Kaplan et al., *J. Biol. Chem.* (1993);268: 13682-13690). The membrane topology of this transporter is similar to that of several other transporters in the inner mitochondrial membrane (Palmieri, *FEBS Lett.* (1994);346: 48-54). In contrast, the plasma membrane NaCT is a much larger protein and its membrane topology, with a predicted eleven transmembrane domains, is different from that of the mitochondrial tricarboxylate transporter. Furthermore, these two transporters also differ in energetics. The mitochondrial tricarboxylate transporter is an electroneutral exchanger and there is no role for a Na^+ gradient in the transport process. In contrast, NaCT is driven by an electrochemical Na^+ gradient. Some microbial

organisms possess a sodium-coupled citrate transporter (van der Rest et al., *J. Biol. Chem.* (1992);267: 8971-8976), but there is no significant structural homology between this transporter and mammalian NaCT.

Is NaCT the Mammalian Ortholog of *Drosophila* Indy? *Indy* is a gene
5 in *Drosophila* which, when made dysfunctional, leads to an extension of the
average life span of the organism (Rogina et al., *Science* (2000);290: 2137-
2140). The putative protein product of this gene is structurally similar to
mammalian NaDC1 and NaDC3 and therefore it was suggested that *Drosophila*
Indy is most likely the species-specific ortholog of either NaDC1 or NaDC3
10 (Rogina et al., *Science* (2000);290: 2137-2140). In Example 1 a functional
clone of *Drosophila* Indy was isolated and its transport function characterized.
Drosophila Indy functions as a Na⁺-independent, electroneutral transporter for a
variety of citric acid cycle intermediates. The functional characteristics of
Drosophila Indy are different from those of NaDC1 and NaDC3. Therefore,
15 neither NaDC1 nor NaDC3 represents the mammalian ortholog of *Drosophila*
Indy. The newly identified NaCT is the newest member of the NaDC/NaSi
gene family in mammals.

NaCT represents the mammalian ortholog of *Drosophila* Indy.
Drosophila Indy transports citrate much more effectively than it does succinate.
20 In this respect, the NaCT resembles *Drosophila* Indy. Furthermore, there is also
significant similarity between the tissue expression pattern of NaCT in
mammals and that of Indy in *Drosophila*. NaCT is expressed abundantly in
mammalian liver, a highly metabolic organ involved in fatty acid and
cholesterol biosynthesis and energy storage. Similarly, in *Drosophila*, Indy is
25 expressed abundantly in the fat body, an organ of metabolic function compared
with that of liver in mammals. However, the two transporters differ in their
energetics. While *Drosophila* Indy is a Na⁺-independent transporter for citrate,
NaCT is a Na⁺-coupled transporter for citrate. Furthermore, *Drosophila* Indy is
electroneutral with no role for membrane potential in the transport process
30 mediated by the transporter. In contrast, NaCT is electrogenic with its transport
function associated with membrane depolarization. Thus, the two transporters
are similar in substrate selectivity and tissue expression pattern but are different
in their transport mechanism.

Example 3

Human Na⁺-coupled Citrate Transporter (NaCT): Primary Structure, Genomic Organization, and Transport Function.

5 This example describes the cloning and functional characterization of the
human Na⁺-coupled citrate transporter (NaCT). The cloned human NaCT
shows 77% sequence identity with rat NaCT. The *nact* gene is located on
human chromosome 17 at p12-13. NaCT mRNA is expressed most
predominantly in the liver, with moderate expression detectable in the brain and
10 testis. When functionally expressed in mammalian cells, human NaCT
mediates the Na⁺-coupled transport of citrate. Studies with several
monocarboxylates, dicarboxylates, and tricarboxylates show that the transporter
is selective for citrate with comparatively several-fold lower affinity for other
intermediates of citric acid cycle. The Michelis-Menten constant for citrate is
15 approximately 650 μ M. The activation of citrate transport by Na⁺ is sigmoidal,
suggesting involvement of multiple Na⁺ ions in the activation process. The
transport process is electrogenic. This represents the first plasma membrane
transporter in humans that mediates the preferential entry of citrate into cells.
Citrate occupies a pivotal position in many important biochemical pathways.
20 Among various citric acid cycle intermediates, citrate is present at the highest
concentrations in human blood. The selectivity of NaCT towards citrate and its
predominant expression in the liver suggest that this transporter may facilitate
the utilization of circulating citrate for the generation of metabolic energy and
for the synthesis of fatty acids and cholesterol.

25 Example 2 described a new transporter from rat brain that recognizes
citrate as the primary substrate was cloned. This transporter, designated as
NaCT (Na⁺-coupled citrate transporter) is structurally homologous to NaDC1
and NaDC3. NaCT transports citrate in a Na⁺-dependent manner and the
transport process is electrogenic. It is the trivalent form of citrate rather than
30 the divalent form that is recognized as the substrate by NaCT. NaCT does
accept succinate as a substrate but with lower affinity compared to citrate. The
tissue expression pattern of NaCT is quite different from that of NaDC1 and

NaDC3. NaCT mRNA is expressed primarily in the liver, testis, and brain in the rat. In this example, the human ortholog of this novel transporter has been is cloned and functionally characterized.

5 MATERIALS AND METHODS

Materials. [^{14}C]-Citrate (sp. radioactivity, 55 mCi/mmol), [^3H]-succinate (sp. radioactivity, 40 Ci/mmol), and [^{14}C]-pyruvate (sp. radioactivity, 15 mCi/mmol) were purchased from Moravsek Biochemicals (Brea, CA). The human retinal pigment epithelial cell line (HRPE), used in heterologous
10 functional expression studies, was maintained in DMEM/F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Cloning of human NaCT from a HepG2 cDNA library. A search of the Genbank database for human established sequence tags (ESTs) with the amino
15 acid sequence of *Drosophila* Indy (Rogina et al., *Science* (2000);290: 2137-2140, Inoue et al., *Biochem. J.* (2002);367: 313-319) as a query identified several ESTs whose predicted amino acid sequences showed significant similarity to that of human NaDC1 and NaDC3. However, there were four ESTs (Genbank Accession Nos. BI490092, BG616615, BI490615, and R01302)
20 with overlapping nucleotide sequences and the predicted amino acid sequence from these ESTs was significantly different from that of human NaDC1 or NaDC3, indicating that this sequence may correspond to a new member of the NaDC gene family. A comparison of this sequence with that of the recently cloned rat NaCT suggested that the new putative transporter is most likely the
25 human ortholog of NaCT. The overlapping nucleotide sequences of these ESTs were used to design primers for RT-PCR to obtain a cDNA probe that is specific for this transporter. The forward primer was 5'-CTCGGCGCTGAGCTATGTCT- 3' (SEQ ID NO:19) and the reverse primer was 5'-GTTGATCTCCGCGAAGG- 3' (SEQ ID NO:20). The predicted size
30 of the RT-PCR product, based on the positions of the primers, was 949 bp. Since these ESTs were identified from a human liver cDNA library and also because NaCT is expressed abundantly in the rat liver, poly(A) RNA from the

human hepatoma cell line HepG2 was used as the template for RT-PCR to obtain the probe. This yielded a cDNA product of expected size. The product was subcloned in pGEM-T vector and sequenced to establish its molecular identity. The cDNA was labeled with [α - 32 P]dCTP by random priming using the ready-to-go oligolabeling beads (Amersham Pharmacia Biotech). This probe was used to screen a HepG2 cDNA library under strong stringency conditions. The screening of the library was done as described previously (Kekuda et al., *J. Biol. Chem.* (1998);273: 15971-15979, Rajan et al., *J. Biol. Chem.* (1999);274: 29005-29010). Positive clones were isolated by secondary or tertiary screening. The library used here was originally established in pSPORT1 vector at *SalI/NotI* site using poly(A) RNA isolated from HepG2 (Hatanaka et al., *Biochim. Biophys. Acta* (2000);1467: 1-6, Hatanaka et al., *Biochim. Biophys. Acta* (2001);1510: 10-17). The cDNA inserts in the vector were under the control of T7 promoter.

DNA sequencing. Sequencing of the sense and antisense strands of the cDNA was performed by *Taq* DyeDeoxy terminator cycle sequencing using an automated Perkin-Elmer Applied Biosystems 377 Prism DNA sequencer. The sequence was analyzed using the National Center for Biotechnology Information server available on the world wide web at ncbi.nlm.nih.gov.

Northern analysis. The expression pattern of NaCT mRNA in a number of human tissues, including brain, colon, heart, kidney, liver, lung, skeletal muscle, placenta, small intestine, spleen, stomach, and testis, was studied by northern analysis using a commercially available human multiple tissue blot (Origene, Rockville, MD). The blot was hybridized with a [32 P]-labeled human NaCT-specific cDNA probe under high stringency conditions. The same blot was then hybridized with a cDNA probe specific for human β -actin as an internal control to detect the presence of RNA in each lane.

Functional expression of human NaCT cDNA in HRPE cells. The functional expression of the cloned transporter was done in HRPE cells using the vaccinia virus expression technique as described previously (Inoue et al., *Biochem. J.* (2002);367: 313-319). Uptake measurements were made at 37° C with radiolabeled citrate, succinate, or pyruvate. The uptake medium in most

experiments was 25 mM Hepes/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, and 5 mM glucose. The time of incubation was 30 minutes for citrate and 15 minutes for succinate and pyruvate. Endogenous uptake was always determined in parallel using the cells transfected with pSPORT1 vector alone. The uptake activity in cDNA-transfected cells was adjusted for the endogenous uptake activity to calculate the cDNA-specific activity. In experiments in which the cation and anion dependence of the uptake process was investigated, NaCl was replaced isoosmotically with LiCl, KCl, *N*-methyl-D-glucamine (NMDG) chloride, and sodium gluconate. Substrate saturation kinetics was evaluated by non-linear regression and the kinetic parameters derived from this method were confirmed by linear regression. In studies involving the dependence of the transport process on Na⁺ concentration, the concentration of Na⁺ was varied by appropriately mixing NaCl and NMDG chloride without altering the osmolality. The influence of membrane potential on the uptake process was investigated by measuring the uptake in the presence of high concentrations of K⁺ (55 mM) in the extracellular medium, a condition that leads to depolarization of the membrane. Experiments were done in duplicate or triplicate and each experiment was repeated two or three times. Results are expressed as means ± S. E.

RESULTS AND DISCUSSION

Structural features of human NaCT cDNA. The human NaCT cDNA (SEQ ID NO:5), also available as GENBANK Accession No. AY151833, is 3,207 bp-long with a poly(A) tail and a 1,707 bp-long open reading frame (including the stop codon). See Fig. 14. The 5'-untranslated region is 12 bp-long and the 3'-untranslated region is 1,488 bp-long. The cDNA possesses a polyadenylation signal (ATTAAA) upstream of the poly(A) tail, which is a variant of the consensus sequence (AATAAA). The cDNA encodes a protein consisting of 568 amino acids (SEQ ID NO:6) (Fig. 14). At the level of amino acid sequence, human NaCT (SEQ ID NO:6) exhibits a high degree of homology to rat NaCT (SEQ ID NO:4) (77% identity, 87% similarity) (Fig. 15).

Human NaCT is however 4 amino acids shorter than rat NaCT. Human NaCT also shows 54% sequence identity with human NaDC1 and 47% sequence identity with human NaDC3. The sequence identity between human NaCT and the human sodium-coupled sulfate transporters NaSi and SUT-1 is

5 comparatively less (43% with human NaSi and 40% with human SUT-1).

Exon-intron organization and chromosomal location of human *nact* gene. A search of the GENBANK database with the nucleotide sequence of human NaCT cDNA as the query identified the chromosomal location and the sequence of the human *nact* gene. The gene is approximately 30 kb in size and
10 consists of at least 12 exons. Fig. 16 describes the exon-intron organization of the human *nact* gene. The translation start site resides in exon 1. Exon 12 codes for a small C-terminal region of the protein and for the entire 3'-untranslated region. The polyadenylation signal is also located in this exon. The sizes of the exons and introns and the sequences at the splice junctions are
15 given in Table 6. The *nact* gene is located on human chromosome 17 p12-13. Interestingly, the human gene coding for NaDC1 is also located on the same chromosome (17p11.1-q11.1) close to the location of the *nact* gene (Pajor, *Am. J. Physiol.* (1996);270: F642-F648). It is also interesting to note that, among the known members of the gene family consisting of the sodium-coupled
20 dicarboxylate and sulfate transporters, human NaCT exhibits the greatest sequence identity with human NaDC1.

Tissue expression pattern of human NaCT mRNA. The expression pattern of NaCT mRNA in human tissues was investigated by northern blot analysis using a commercially available multiple human tissue blot. NaCT
25 mRNA (approximately 3.2 kb) is expressed in a restricted manner in human tissues. The expression is evident only in the liver, testis, and brain. The level of expression in the liver is several-fold higher than in the brain and testis. Kidney and heart show weak, but detectable, hybridization signals. This tissue expression pattern of NaCT is different from that of NaDC1 and NaDC3 (Pajor,
30 *J. Membrane Biol.* (2000);175: 1-8). NaDC1 mRNA is expressed mostly in the small intestine and kidney whereas NaDC3 mRNA is expressed primarily in the kidney, small intestine, liver, placenta, and brain.

Functional features of human NaCT. The functional characteristics of the cloned human NaCT were investigated using a heterologous expression system in a mammalian cell line (HRPE). The expression of the heterologous cDNA was carried out using the vaccinia virus expression technique. The ability of human NaCT to transport succinate, citrate, and pyruvate in the presence of NaCl was first tested (Fig. 17A). The uptake of citrate (20 μ M) in cells transfected with human NaCT cDNA was about 23-fold higher than in cells transfected with vector alone. In contrast, the uptake of succinate (80 nM) and pyruvate (100 μ M) was increased only by 20-30% in cDNA-transfected cells compared to control cells. These data show that human NaCT accepts citrate as the most preferred substrate. Succinate, the prototypical substrate for human NaDC1 and NaDC3, is not recognized as well by human NaCT. The recently cloned rat NaCT also prefers citrate as a substrate, but is able to mediate the uptake of succinate and pyruvate to a much greater extent compared to human NaCT. This indicates that human NaCT exhibits comparatively greater selectivity towards citrate than the rat ortholog. Nonetheless, based on the substrate selectivity, rat NaCT as well as human NaCT can be classified as tricarboxylate transporters rather than dicarboxylate transporters.

Since human NaCT was able to transport citrate preferentially, this tricarboxylate was used as the substrate for subsequent functional characterization of the transporter. The cDNA-mediated uptake of citrate was linear even up to 45 minutes (Fig. 17B). Therefore, all subsequent studies were carried out with a 30-minute incubation. The involvement of Na^+ in the uptake process mediated by human NaCT was evaluated by monitoring the uptake of citrate in vector-transfected cells and in human NaCT cDNA-transfected cells in the presence and absence of Na^+ . This was done by isoosmotically replacing NaCl in the uptake medium with NMDG chloride, KCl, and LiCl (Table 7). The cDNA-specific uptake was completely abolished when Na^+ was substituted with other monovalent cations. The uptake process was however not dependent on Cl^- because replacement of Cl^- with gluconate had no effect on the cDNA-specific citrate uptake.

The substrate specificity of human NaCT was examined by competition studies in which the ability of various unlabeled compounds (2.5 mM) to compete with [14 C]-citrate (20 μ M) for uptake via human NaCT was assessed (Table 8). Unlabeled citrate was the most potent inhibitor of [14 C]-citrate uptake mediated by human NaCT. Among the dicarboxylates, only succinate and malate showed significant inhibitory effect. Fumarate, α -ketoglutarate, and maleate were unable to inhibit the uptake of [14 C]-citrate. The monocarboxylates pyruvate and lactate were also not effective in inhibiting [14 C]-citrate uptake. Interestingly, isocitrate and *cis*-aconitate, close structural analogs of citrate, were also excluded as substrates by human NaCT. The affinities of human NaCT for citrate and dicarboxylates (succinate, malate, and fumarate) was then compared by studying the dose-response relationship for the inhibition of [14 C]-citrate uptake mediated by human NaCT. The IC_{50} value (i.e., concentration of the inhibitor necessary for 50% inhibition) calculated for citrate from the dose-response relationship was 688 ± 150 μ M. The corresponding value for succinate, malate, and fumarate were 1.92 ± 0.48 , 3.12 ± 1.34 , and 12.6 ± 5.0 mM, respectively. These values for the dicarboxylates are several-fold higher than that for citrate. These results show that, among the various intermediates of the citric acid cycle, citrate is the most preferred substrate for human NaCT.

Kinetic features of human NaCT. Citrate uptake mediated by human NaCT was saturable with a K_t of 604 ± 73 μ M (Fig. 18A). Interestingly, this value is markedly different from the corresponding value for rat NaCT. In the case of rat NaCT, the K_t value for citrate is 18 ± 4 μ M. Thus, the affinity of human NaCT for citrate is about 30-fold less than the affinity of rat NaCT for citrate under identical assay conditions. Kinetic analysis of Na^+ -activation of citrate uptake via human NaCT showed that the uptake process is obligatorily dependent on Na^+ . However, the activation failed to reach the maximum within the physiological concentrations of Na^+ (Fig. 18B). Nonetheless, the activation response was not hyperbolic in nature. It was sigmoidal, indicating the involvement of multiple Na^+ ions in the activation process. Since the activation did not reach the maximum within the Na^+ concentration range tested, the exact

number of Na^+ ions involved in the activation process could not be determined. The concentration range could not be extended beyond 140 mM due to the fact that the extracellular medium would become hyperosmolar when the concentration of NaCl goes beyond 140 mM. But, the inability of Na^+ to saturate the uptake process within the physiological concentrations indicates that the transporter exhibits low affinity for Na^+ . This characteristic of human NaCT differs from that of rat NaCT. The uptake of citrate mediated by rat NaCT under similar experimental conditions is activated to the maximum within the physiological concentrations of Na^+ . Since the activation reaches the maximum, the Hill coefficient for Na^+ -activation could be calculated for rat NaCT. The value is between 3 and 4. It can be speculated that the Na^+ :citrate stoichiometry for rat NaCT is 4:1. Such a stoichiometry will render the uptake process mediated by rat NaCT electrogenic. This was indeed the case because membrane depolarization was able to inhibit the uptake of citrate mediated by rat NaCT. It is predicted that human NaCT also behaves in a similar manner with respect to the coupling of citrate uptake with Na^+ . This is supported by the electrogenic nature of the transport process mediated by human NaCT. The cDNA-specific citrate uptake between control conditions (concentration of K^+ in the extracellular medium, 5 mM) and membrane-depolarizing conditions (concentration of K^+ in the extracellular medium, 55 mM) was compared. The uptake was inhibited significantly ($58 \pm 2\%$) when the membrane was depolarized, indicating that the uptake process is influenced by membrane potential. Since depolarization inhibits the uptake, it can be concluded that the uptake process mediated by human NaCT is electrogenic, associated with a net transfer of positive charge into the cells. Therefore, the Na^+ :citrate stoichiometry is 4:1 for human NaCT as is the case for the rat ortholog.

In summary, a human transporter (NaCT) that mediates the cellular entry of citrate by a process energized by the electrochemical Na^+ gradient has been cloned and characterized. This represents the first human plasma membrane transporter with preferential selectivity towards citrate as a substrate.

TABLE 6
Exon-intron boundaries of the human *nact* gene

5' Exon			Intron			3' Exon	
Number	Size (bp)	Sequence	Donor	Size (bp)	Number	Acceptor	Sequence
1	>114	..GCCAAG	gtcagt...	6075	1	...cttcag	TTTGTC...
2	129	..AGGCAG	gtgagc...	249	2	...ctccag	GTGTGT...
3	137	...TGCACG	gtaatt...	2585	3	...ctgcag	GCTGAT...
4	179	...TGCCAG	gtgagc...	739	4	...ccacag	GGAGTC...
5	169	...GAACGA	gtgagt...	1843	5	...ttgtag	GTTGTT...
6	123	...ATTCAA	gtaagt...	5062	6	...ctccag	TTTTAA...
7	216	...GACAAA	gtaagt...	1528	7	...acctag	GTATGT...
8	101	...AGGAAG	gtaagt...	934	8	...tcccag	AAAGGA...
9	119	...TCCGAG	gtaagt...	2103	9	...tcccag	GCCTCG...
10	162	...TCCATG	gtaagt...	3112	10	...ccgcag	TCTCGC...
11	138	...GACATG	gtaaca...	1190	11	...ttccag	GTGAAA...
12	1617	...CCGCGG					

TABLE 7

Ion dependence of citrate uptake via human NaCT

Uptake of [^{14}C]-citrate (20 μM) was measured in vector-transfected HRPE cells and in human NaCT cDNA-transfected HRPE cells at pH 7.5 in the presence of various inorganic salts. Values represent means \pm S.E.

Salt	Citrate Uptake			
	Vector	CDNA	cDNA-specific	
	pmol/ 10^6 cells/min			%
NaCl	1.5 ± 0.3	59.9 ± 3.9	58.4 ± 3.9	100
NMDG chloride	1.4 ± 0.2	1.3 ± 0.2	-0.1 ± 0.2	0
KCl	1.2 ± 0.2	1.1 ± 0.1	-0.1 ± 0.1	0
LiCl	1.3 ± 0.1	2.0 ± 0.1	0.7 ± 0.1	1
Sodium gluconate	1.6 ± 0.3	61.7 ± 4.7	60.1 ± 4.7	103

TABLE 8

Substrate specificity of human NaCT

Uptake of [^{14}C]-citrate (20 μM) was measured in vector-transfected HRPE cells and in human NaCT cDNA-transfected HRPE cells in the absence or presence of various monocarboxylates, dicarboxylates or tricarboxylates (2.5 mM). Data (means \pm S.E.) represent only cDNA-specific uptake.

Unlabeled compound	[^{14}C]-Citrate Uptake	
	pmol/ 10^6 cells/min	%
None	47.7 ± 4.8	100
Citrate	7.7 ± 0.7	16
Succinate	32.1 ± 3.6	67
Malate	29.5 ± 1.9	62
Fumarate	43.7 ± 5.0	92
α -Ketoglutarate	52.9 ± 3.3	111
Maleate	58.0 ± 6.7	121
Pyruvate	51.5 ± 3.8	108
Lactate	61.2 ± 6.0	128
Malonate	57.3 ± 3.2	120
Isocitrate	59.6 ± 1.1	125
<i>cis</i> -Aconitate	55.5 ± 3.3	116

Example 4

Functional Characterization of a Sodium-coupled Citrate Transporter from *Caenorhabditis elegans* and the Relevance of the Transporter to Body Fat Storage and Life Span

5

Na⁺-coupled citrate transporter (ceNaCT) from *C. elegans* has been cloned and functionally characterized. This transporter shows significant sequence homology with the *Drosophila* Indy and the mammalian Na⁺-coupled citrate transporter NaCT. When heterologously expressed in a mammalian cell
10 line or in *Xenopus* oocytes, the cloned ceNaCT mediates the Na⁺-coupled transport of various intermediates of the citric acid cycle. The substrates for ceNaCT include dicarboxylates such as succinate as well as the tricarboxylate citrate. Monocarboxylates are excluded by the transporter. The substrate specificity of this transporter is different from that of the previously identified
15 ceNaDC1 and ceNaDC2. The transport process is electrogenic as evidenced from the substrate-induced inward currents in oocytes expressing the transporter under voltage-clamp conditions. The *nact* gene is expressed more predominantly during the early stages of development than during adult life of the organism. Tissue specific expression pattern studies using a reporter gene
20 fusion method in transgenic *C. elegans* show that the gene is expressed in the intestinal tract, the organ responsible for not only the digestion and absorption of nutrients but also for the storage of energy in this organism. Functional knockdown of the transporter by RNA interference (RNAi) not only leads to a significant increase in life span, but also causes a dramatic decrease in fat
25 storage in the intestinal tract. Citrate occupies a pivotal position in metabolic energy production and in the synthesis of fatty acids and cholesterol and the present data show that the newly cloned transporter plays an obligatory role in the cellular utilization of extracellular citrate for these metabolic functions. Since mammals express an ortholog of ceNaCT in the liver, an organ involved
30 in fatty acid and cholesterol synthesis, the data from the present study suggest that the transporter may play a similar role in the mammalian liver.

Even though the mammalian NaCT is similar to drIndy with respect to the recognition of citrate as a substrate, NaCT differs from drIndy in terms of

Na⁺-dependence. This example reports the molecular identification of the NaCT ortholog in *C. elegans*, its structural and functional characteristics, and its relevance to life span. As shown in this example, *C. elegans* NaCT is a Na⁺-coupled transporter that recognizes citrate as a substrate. RNAi-based
5 knockdown of NaCT function leads to a significant increase in the life span of this organism. In *C. elegans*, NaCT is expressed primarily in the intestinal tract, an organ with metabolic function similar to that of the liver in mammals. Furthermore, since citrate plays a pivotal role in the synthesis of fatty acids and cholesterol, it was investigated whether the transporter has any functional
10 relevance to the utilization of extracellular citrate in these metabolic functions. This example also shows that RNAi-based knockdown of NaCT function in *C. elegans* leads to a marked decrease in the levels of fat stores in the intestinal tract.

15 EXPERIMENTAL PROCEDURES

Materials. [¹⁴C]Citrate (specific radioactivity, 55 mCi/mmol) and [³H]succinate (specific radioactivity, 40 Ci/mmol) were purchased from Moravsek Biochemicals (Brea, CA). The human retinal pigment epithelial (HRPE) cell line, used routinely for heterologous expression of cloned
20 transporters, was maintained in Dulbecco's minimum essential medium/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Lipofectin was purchased from Invitrogen. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Magna nylon transfer membranes used in library screening were purchased
25 from Micron Separations (Westboro, MA). Unlabeled monocarboxylates, dicarboxylates, and ticarboxylates were obtained from Sigma.

Nematode culture and RNA preparation. A wild type nematode strain, *C. elegans* N2 (Bristol) was obtained from the *Caenorhabditis* Genetics Center (St. Paul, MN). Nematode culture was carried out using a standard procedure
30 with a large-scale liquid cultivation protocol (Fei et al., *J Biol Chem* (2003); 278: 6136-6144; Wood, (1988) in The nematode *Caenorhabditis elegans*. (Wood, W.B and the Community of *C. elegans* Researchers, eds) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 587-606; Fei et al., *Biochem*

J (1998);332: 565-572; and Fei et al., *J Biol Chem* (2000);275: 9563-9571).

The nematodes were cleaned by sedimentation through 15% (w/v) Ficoll 400 in 0.1 M NaCl. The pellet was then used for total RNA preparation. Total RNA was isolated using the TRIzol reagent (GIBCO-BRL, Gaithersburg, MD).

5 Poly(A)⁺ mRNA was purified by affinity chromatography using oligo(dT)-cellulose.

Reverse transcription polymerase chain reaction (RT-PCR) and hybridization probe preparation. A search in the WormBase (Release WS93 on the worldwide web at wormbase.org) using the drIndy protein sequence as a query revealed that the gene R107.1 located on chromosome III encodes a
10 hypothetical sodium-coupled transporter belonging to the family of sodium-coupled dicarboxylate transporters. The putative protein product of this gene is different from the previously identified NaDC1 and NaDC2 in this organism. This indicated that this gene is a potential candidate for NaCT in *C. elegans*. A
15 pair of PCR primers specific for this gene was designed based on the sequence of the cosmid R107.1 (GenBank accession no. Z14092): forward primer, 5'-CTC CAT CGA AGA ATC GCA C-3' (SEQ ID NO:21) and reverse primer, 5'-GAA ATA GCA TAC CCA GCA CC-3' (SEQ ID NO:22). These primers yielded a single RT-PCR product with RNA from *C. elegans*. The size of the
20 product was approximately 1.0 kb which agreed with the predicted size based, on the distance between the two primers in the theoretically derived gene transcript. The RT-PCR product was gel-purified and subcloned into pGEM-T easy vector (Promega Madison, WI). The molecular identity of the insert was established by sequencing. This cDNA fragment was used as a probe to screen
25 a *C. elegans* cDNA library.

Construction of a directional *C. elegans* cDNA library. SuperScript Plasmid System from GIBCO-BRL (Gaithersburg, MD) was used to establish the cDNA library using the poly(A)⁺ RNA from *C. elegans*. The transformation of the ligated cDNA into *E. coli* was performed by electroporation using
30 ElectroMAX DHIOB competent cells. The bacteria plating, the filter lifting, the DNA fragment labeling, and the hybridization methods followed the routine procedure (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 9.31-

9.50). The DNA sequencing of the full-length ceNaCT cDNA clone was performed using an automated Perkin-Elmer Applied Biosystems 377 Prism DNA sequencer (Foster City, CA) and the Taq DyeDeoxy terminator cycle sequencing protocol.

5 Vaccinia/T7 expression system. Functional expression of the ceNaCT cDNA in HRPE cells was done using the vaccinia virus expression system as described previously (Fei et al., *J Biol Chem* (2003);278: 6136-6144, Kekuda et al., *J Biol Chem* (1999);274: 3422-3429, Huang et al., *J Pharmacol Exp Ther* (2000);295: 392-403, Wang et al., *Am J Physiol Cell Physiol* (2000);278: C1019-1030, Inoue et al., *Biochem J*, (2002);367: 313-319, Inoue et al., (2002) *J Biol Chem* (2002);277: 39469-39476, Inoue et al., (2002) *Biochem Biophys Res Commun* (2002);299: 465- 471). HRPE cells grown in 24-well plates were infected with a recombinant vaccinia virus (VTF₇₋₃) at a multiplicity of 10 plaque-forming units/cell. The virus was allowed to adsorb for 30 minute at 37° C with gentle shaking of the plate. Cells were then transfected with the plasmid DNA (empty vector pSPORT or ceNaCT cDNA constructs) using the lipofection procedure (GIBCO-BRL, Gaithersburg, MD). The cells were incubated at 37° C for 12 hours and then used for determination of transport activity. Uptake of [¹⁴C]-citrate and [³H]-succinate was determined at 37° C as described previously (Inoue et al. (2002) *Biochem J* (2002);367: 313-319, Inoue et al., (2002) *J Biol Chem* (2002);277: 39469-39476, Inoue et al., (2002) *Biochem Biophys Res Commun* (2002);299: 465- 471). In most experiments, the uptake medium was 25 mM Hepes/Tris (pH 7.5) or 25 mM Mes/Tris (pH 6.5), containing 140, mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, and 5 mM glucose. In experiments in which the cation and anion dependence of the transport process was investigated, NaCl was replaced isoosmotically by LiCl, KCl, sodium gluconate, or N-methyl-D-glucamine (NMDG) chloride. Uptake measurements were routinely made in parallel in control cells transfected with the plasmid alone and in cells transfected with the vector-cDNA construct. The uptake activity in cDNA-transfected cells was adjusted for the endogenous activity measured in control cells to calculate the cDNA-specific activity. Experiments were performed in triplicate and each experiment was repeated at least three times. Results are presented as means ±S.E.

Electrophysiological studies of ceNaCT transport activity. Initially, ceNaCT cRNA prepared from the pSPORT-ceNaCT cDNA construct was used for functional expression of the transporter in *X. laevis* oocytes. These initial attempts were unsuccessful. Therefore, a *X. laevis* oocyte expression vector was used. The coding sequence of ceNaCT cDNA was amplified by PCR using the following primers: 5'-CCC GGG TAT GAA GCC TAG CCC CCA GCG TAC GTT AAT AAA A-3' (SEQ ID NO:23) (forward primer) and 5'-GCG GAT CCA AAA ATT AGC AAA CTG GAT ATG AAG AGT TTT CTG AAG-3' (SEQ ID NO:24) (reverse primer). The forward primer contained the start codon and also an *Xma I* site introduced at the 5'-end for the purpose of subcloning. The reverse primer contained the termination codon and also a *BamH I* site at the 5'end for the purpose of subcloning. The PCR-derived ceNaCT coding fragment was then inserted into the *Xma I/BamH I* site in the oocyte expression vector pGHI9. In this construct, the ceNaCT coding sequence was flanked by a synthetic *Xenopus* (β -globin gene 5'-UTR and 3'-UTR regulatory elements. Plasmid pGHI9-ceNaCT cDNA was linearized with *Xho I* and transcribed *in vitro* using the mMMESSAGE mMACHINE RNA transcription kit (Ambion, Austin, TX). When the cRNA derived from the pGHI9-ceNaCT cDNA construct was injected into the oocytes, the expression of ceNaCT was detected by monitoring the uptake of the [14 C]citrate. The procedures for oocyte manipulation, microinjection and electrophysiological studies using the two-electrode voltage-clamp (TEVC) protocol have been described previously (Fei et al., (1998) *Biochem J* 332, 565-572, Fei et al. (2000) *J Biol Chem* 275, 9563-9571, Fei et al., (1994) *Nature* 368, 563-566, Mackenzie et al., (1996) *Biochim BiophysActa* 1284, 125-128).

Semi-quantitative RT-PCR. An RT-PCR assay with the ceNaCT-specific primers described in the previous section was used to study the developmental stage-specific expression pattern of the *nact* gene. A Quantum RNA 18S internal standard (Ambion, Austin, TX) was used for the semi-quantitative RT-PCR. Total RNA (approximately 1.0 μ g), isolated from different developmental stages of *C. elegans* (embryo, early larva, late larva, and adult), was taken as template to perform reverse transcription using an RT-PCR kit from Perkin Elmer Corp. (Norwalk, CT) as described previously (Fei et

al., (2003) *J Biol Chem* 278, 6136-6144, Fei et al., (1998) *Biochem J* 332, 565-572). Reverse transcription was followed by PCR in a multiplex format. The gene specific primers and the primers for the internal control (18S rRNA) with their competitors were combined at a predetermined ratio. The resultant
5 multiplex PCR products were subjected to electrophoresis on an 1 % agarose gel and the steady state levels of ceNaCT mRNA at different developmental stages were estimated from the relative ratios of the intensity of the ceNaCT specific RT-PCR product to the intensity of the 18S rRNA-specific RT-PCR product at each of these stages.

10 Analysis of tissue-specific expression pattern of ceNaCT. To study the tissue-specific expression pattern of the *nact* gene in *C. elegans*, a transcriptional *nact::gfp* fusion gene was constructed and transgenic animals expressing these transgenes were developed as described previously (Fei et al., (2003) *J Biol Chem* 278, 6136-6144, Fei et al. (2000) *J Biol Chem* 275, 9563-
15 9571). A GFP-expression vector pPD 117.01 was a gift from Dr. A. Fire (Carnegie Institution of Washington, Baltimore, MD). The cosmid R107 containing the *C. elegans nact* gene and its promoter was obtained from the Sanger Center (Cambridge, UK). A DNA fragment containing the *C. elegans nact* gene promoter region was generated by PCR using this cosmid DNA as the
20 template. The sense primer (coordinates in cosmid R107 are 11,660-11,630) with a *Sal* I adapter attached to the 5'-end was 5'-GTC GAC GAG GTG TTA AAC TGT ATA GTC GTG GTG-3' (SEQ ID NO:25) and the reverse primer (coordinates in cosmid R107 are 9,609-9,637) with a *Bam*HI adapter attached to the 5'-end was 5'-GCC GGA TCC AAG AAG TAC CAG AAG CTT TTT
25 TAT-3' (SEQ ID NO:26). A recombinant KlenTaq DNA-polymerase (AB Peptides, St. Louis, MO) was used for the long-range PCR. The size of the PCR product was ~2.1 kb. The PCR product was digested with *Sal* I and *Bam*HI and ligated into the multiple cloning site II in the pPD117.01 vector. Bacterial transformation and plasmid preparation followed a standard protocol (Sambrook
30 et al., (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 9.31-9.50). The minigene fusion constructs were verified by sequencing. Transgenic lines were established using a standard germ line transformation protocol (Mello and Fire,

Methods Cell Biol (1995);48: 451-482). A cloned mutant collagen gene containing the *rol-6* (plasmid pRF4) was used as a dominant genetic marker for DNA transformation (Kramer et al., *Mol Cell Biol* (1990);10: 2081-2089, Pettitt and Kingston, *Dev Biol* (1994);161: 22-29). The F1 rollers were picked up
5 according to their characteristic rolling behavior and cultured individually to establish transformed lines. GFP expression pattern was determined by fluorescence microscopy (Chalfie et al., *Science* (1994);263: 802-805 and Miller and Shakes, *Methods Cell Biol* (1995);48 :365-394).

Bacteria-mediated RNA interference (RNAi) and life span measurement.
10 A fragment of the coding region of ceNaCT cDNA was generated by PCR and subcloned into a “double T7” plasmid pPD129.36, which was subsequently transformed into HT115 (DE3) cells. Induction of HT115 cells harboring the double-T7 plasmid to express dsRNA and the bacteria-mediated RNAi procedure were carried out as previously described (Fei et al., *J Biol Chem*
15 (2003);278: 6136-6144; Fire et al., *Nature* (1998);391: 806-811); and Timmons et al., *Gene* (2001);263: 103-112).

Mean life spans from different groups were compared using the nonparametric log-rank analysis. The survival curves were plotted according to the Kaplan-Meier algorithm using Minitab software (version 13, Minitab Inc.
20 State College, PA). Nematode body size measurement was made on day 5. Worms were placed on agar plates and anesthetized using 0.1 M NaN₃. To facilitate measurement, animals were laid out straight using a platinum wire. Measurement was carried out under a dissecting microscope, with an eyepiece graticule calibrated with a stage micrometer (Olympus, Melville, NY). To
25 calculate body volume, worms were treated as cylinders ($V=\pi(1/2D)^2L$), where D is the body width and L is body length.

To serve as a positive control for the bacteria-mediated RNAi in the assessment of the influence of ceNaCT on life span, an HTI 15 strain containing DAF-2specific dsRNA was included. Knockdown of DAF-2 function in *C.*
30 *elegans* leads to an increase in life span in this organism (Kenyon et al. (1993) *Nature* 366, 461-464, Wolkow et al., (2000) *Science* 290,147-150). Life span of age-synchronous nematodes was determined at 20° C as described previously (Fei et al., (2003) *J Biol Chem* 278, 6136-6144). To avoid any potential

subjective errors in the determination of life span, the studies were conducted in a blinded manner in which the person performing the life span analysis was unaware of the identity of the dsRNA used in feeding the nematodes. Statistical analysis was performed using the Microsoft EXCEL 2000 analysis ToolPak.

- 5 Mean life spans from different groups were compared using the Student's t-test assuming unequal population variances. The survival curves were plotted according to the Kaplan-Meier algorithm using Sigma Plot (version 8.0, SPSS Inc., Chicago IL).

Quantitative fat deposit analysis by laser-scanning confocal microscopy.

- 10 Fat storage droplets in living *C. elegans* were visualized using the vital dye Nile red (Molecular Probes, Eugene, OR). This dye is a selective stain for intracellular lipid droplets (Greenspan et al., *J Cell Biol* (1985);100:965-973). Addition of Nile red to *E. coli*, the common laboratory diet of *C. elegans*, resulted in uptake and incorporation of the dye into lipid droplets in intestinal
- 15 cells, the principal location of fat storage in this organism. It is known that Nile red staining does not affect animals' growth rate, brood size, feeding or life span (Ashrafi et al., *Nature* (2003);421: 268-272). Nile red stock solution (500 µg/ml) was prepared in acetone and was diluted in phosphate buffered saline to a final concentration of 0.1 µg/ml (working solution). Eggs, obtained from
- 20 gravid hermaphrodites using an alkaline hypochlorite treatment procedure, were dispensed on NGM (Nematode Growth Medium) petri dishes with bacteria lawn and allowed to hatch. The IPTG-induced HT 115 bacteria suspension harboring different gene-specific dsRNAs was mixed with an equal volume of the Nile red working solution and fed to the newly hatched worms on individual petri dishes
- 25 every day until the worms developed into the adult stage (Fei et al., *J Biol Chem* (2003);278: 6136-6144). Nile redstained worms were placed on 2% agarose pads attached to microscope slides and anesthetized with 0.1 M sodium azide for examination by confocal fluorescence microscope (Zeiss Axioplan2 upright microscope equipped with a Zeiss 510 NLO scan head (Carl Zeiss
- 30 Microlmaging, Inc., Thornwood, NY). All Nile red images were acquired using identical settings and exposure times. The excitation filter wavelength was 515-560 nm and the emission filter wavelength was >590 nm. Fluorescence intensity was quantified on equal planes (optical section thickness <100 µm),

which encompassed the entire worm body. The LSM 510 software (version 3.0 SP3, Carl-Zeiss, Heidelberg, Germany) was used to calculate the total fluorescence intensity for all Nile red stained droplets within the animal body as the product of area multiplied by mean fluorescence. At least 10-15 animals in each group were examined for fluorescence intensity measurements and average intensity for each tested group was compared using a Student's t-test (Sigma Plot version 8.0).

RESULTS

Molecular cloning and structural characterization of ceNaCT. The *C. elegans nact* gene is localized on chromosome III and its size is at least approximately 2.7 kb, excluding the promoter region. The gene consists of 11 exons as deduced by a comparison between the sequence of the cloned cDNA with that of the GenBank deposit R107.1 from the nematode genome sequence project (WormBase release WS93). The structural organization of the gene is shown in Fig. 20. The ceNaCT cDNA is 1,747 bp long and contains a poly(A) tail. The 5'- and the 3'-untranslated regions are 30 by and 44 by long, respectively. The ceNaCT protein deduced from the cDNA sequence contains 551 amino acids with an estimated molecular size of 61 kDa and an isoelectric point of 7.24. According to the Kyte-Doolittle hydropathy plot, the ceNaCT protein possesses 12 putative transmembrane domains. The ceNaCT cDNA and its encoded transporter protein sequences have been deposited into the GenBank (Accession number: AY090486).

A pair-wise comparison analysis of the transporter protein sequences between ceNaCT and its closely related functional counterparts, the mammalian NaCT and the *Drosophila* Indy, using the BESTFIT algorithm in the GCG package (version 10.2, Madison WI) has shown that ceNaCT is closely related to mammalian NaCT (49% similarity and 36% identity) and to drIndy (48% similarity and 35% identity). The mammalian NaCT and drIndy are also similar to each other (51% similarity and 37% identity). Following a multiple protein sequence alignment of the three transporters, using the PILEUP program and in combination with the PRETTYBOX program in the GCG package, a sodium symporter family signature motif was identified within these transporter

proteins (Fig. 21). A consensus pattern established for the signature sequence is: (S)SXXFXXP(V)(G)XXXNX(I)V (SEQ ID NO:29), wherein X denotes any amino acid residue, (S) denotes serine or other related amino acids, such as alanine, cysteine, threonine, or proline, (V) denotes valine or other related amino acids, such as leucine, isoleucine or methionine, (G) denotes glycine or other related amino acids, such as serine or alanine, and (I) denotes isoleucine or other related amino acids, such as leucine, valine, or methionine. This sodium symporter family is a group of integral membrane proteins that mediate the cellular uptake of a wide variety of molecules including di- or tri-carboxylates and sulfate by a transport mechanism involving sodium cotransport (Pajor, *Annu Rev Physiol* (1999);61: 663-682 and Pajor, *J Membr Biol* (2000);175: 1-8).

Functional characterization of ceNaCT using a heterologous mammalian expression System. The functional analysis of the cloned ceNaCT was carried out by heterologous expression of the cDNA in HRPE cells using the vaccinia virus expression system. Since the ceNaCT transporter protein is structurally similar to drlndy and mammalian NaCT, which preferentially recognize citrate as a substrate, the transport function of ceNaCT with citrate as a potential substrate was tested. The uptake of [¹⁴C]citrate (10 μM) in the presence of extracellular Na⁺ (pH7.5) increased approximately 45-fold in cells expressing ceNaCT (61 ± 5 pmol/10⁶ Cells/min) compared to vector-transfected control cells (1.3±0.1 pmol/10⁶ cells/min). Then, the ability of ceNaCT to transport the dicarboxylate succinate was tested. Surprisingly, the uptake of succinate was also increased markedly in ceNaCT-expressing cells compared to control cells. However, the ability of ceNaCT to transport citrate was much greater than to transport succinate, when measured under identical conditions (10 μM substrate concentration). Since it has already been shown that succinate is a substrate for the previously cloned ceNaDC1 and ceNaDC2, the abilities of all three transporters to transport citrate and succinate under identical conditions was compared. As shown earlier, ceNaDC1 and ceNaDC2 were able to transport succinate very effectively (Fig. 22A). The magnitude of succinate transport was comparable between ceNaDC2 and ceNaCT whereas that of ceNaDC1 was comparatively a little lower. In contrast, the magnitude of citrate transport was

the highest for ceNaCT. ceNaDC2 showed only a minimal ability to transport this tricarboxylate. The ability of ceNaDC1 to transport citrate was much higher than that of ceNaDC2 but much lower than that of ceNaCT. These data show that, among these three transporters, ceNaCT is the only transporter that
5 recognizes citrate as the preferred substrate. The ceNaCT-mediated citrate uptake was obligatorily dependent on the presence of Na⁺ because substitution of Na⁺ with Li⁺, K⁺, or NMDG (*N*-methyl-D-glucamine) abolished completely the cDNA-induced increase in citrate uptake. There was no involvement of chloride in the uptake process as indicated by comparable uptake activities in
10 the presence of NaCl or sodium gluconate (Fig. 22B).

The substrate specificity of ceNaCT was studied using a competition analysis by monitoring the ability of various monocarboxylates, dicarboxylates, and tricarboxylates (2.5 mM) to compete with [¹⁴C]citrate (15 μM) for the uptake process. Uptake measurements were made in parallel in vector-
15 transfected cells and in cDNA-transfected cells and the cDNA-specific uptake was calculated by subtracting the uptake in vector-transfected cells from the uptake in cDNA-transfected cells. Only the cDNA-specific uptake was used in the analysis. Unlabeled citrate was a potent inhibitor of [¹⁴C] citrate uptake (Fig. 22C). Interestingly, the two other tricarboxylates tested, namely isocitrate
20 and cis-aconitate, did not compete with [¹⁴C]citrate as effectively as unlabelled citrate. Among the various dicarboxylates tested, the ceNaCT-mediated citrate uptake was inhibited markedly by succinate, α-ketoglutarate, fumarate and malate. In contrast to fumarate, its stereoisomer maleate failed to compete with citrate. Similarly, malonate, a structural homolog of succinate also failed to
25 inhibit the uptake of citrate. The monocarboxylates, pyruvate and lactate, caused only a minimal inhibition.

Citrate uptake mediated by ceNaCT was influenced by extracellular pH (Fig. 22D). The uptake increased markedly when the extracellular pH was changed from 7.5 to 6.5. Further acidification of extracellular pH resulted in a
30 decrease of uptake. The uptake activity at pH 6.5 was 3- to 4-fold higher than that at pH 7.5. These data could be interpreted in two ways. The uptake activity in the pH range 8.0-6.5 might indicate that citrate is recognized by ceNaCT as a substrate only in its dianionic form. Alternatively, the pH-

dependence may simply indicate the pH optimum for the catalytic function of the transporter. To differentiate between these two possibilities, the pH-dependence of succinate uptake via ceNaCT in parallel was studied (Fig. 22D). Succinate exists predominantly in its dianionic form over the pH range 6.5 - 8.0; yet the uptake of succinate via ceNaCT was also enhanced when the pH was changed from 8.0 to 6.5. This indicates that the pH-dependence represents the pH optimum for the transporter and that citrate is likely to be recognized as a substrate in its trivalent form. This would mean that ceNaCT is able to recognize a dicarboxylate as well as a tricarboxylate as substrates. Fig. 22D also shows that, under identical conditions, the transport rate via ceNaCT is much higher for citrate than for succinate over the entire pH range studied (5.5-8.0). The transport of citrate via ceNaCT was saturable with a Michaelis-Menten constant (K_t) of $76 \pm 14 \mu\text{M}$ at pH 7.5 (Fig. 23A). The corresponding value for succinate was $88 \pm 13 \mu\text{M}$ under identical conditions (Fig. 23B). These data show that ceNaCT is a high-affinity Na^+ -coupled transporter for the tricarboxylate citrate and the dicarboxylate succinate. Interestingly, even though the affinities for citrate and succinate are comparable, the transport rate for citrate is much greater than for succinate, suggesting that the transport processes of these two substrates differ in maximal velocity (V_{max}). This is evident from the V_{max} values for these two substrates ($270 \pm 13 \text{ pmol}/10^6 \text{ cells}/\text{min}$ for citrate versus $210 \pm 8 \text{ pmol}/10^6 \text{ cells}/\text{min}$ for succinate). These characteristics are different from those of ceNaDC1 and ceNaDC2. Thus, the transport features of ceNaCT are similar to those of *Drosophila* Indy. However, ceNaCT and *Drosophila* Indy differ in their dependence on Na^+ . ceNaCT is also similar to the recently identified mammalian NaCT not only in Na^+ -dependence but also in the ability to transport citrate much more effectively than succinate.

The effect of Na^+ on the uptake of citrate and succinate was then investigated by measuring the uptake in the presence of varying concentrations of extracellular Na^+ in cells transfected with either ceNaCT eDNA or plasmid alone. Again, the uptake values were adjusted for the endogenous uptake activity measured under identical conditions in cells transfected with vector alone. The concentration of Na^+ in the uptake medium was varied from 0-140

mM. The osmolality of the medium was maintained by adding appropriate concentrations of NMDG chloride as a substitute for NaCl. The relationship between the cDNA-specific uptake and Na^+ concentration was sigmoidal for citrate as well as succinate, suggesting the involvement of more than one Na^+ per substrate molecule transported.

Functional characterization of ceNaCT using the *X. laevis* oocyte expression system. ceNaCT mediates the Na^+ -coupled transport of several dicarboxylates' as well as the tricarboxylate citrate. To determine whether the transport process is electrogenic, the *X. laevis* oocyte expression system was used to express ceNaCT heterologously. This system is amenable to study the electrogenic characteristics of transport processes by using the two-electrode voltage clamp technique. First, it was tested whether the transporter is expressed in oocytes injected with ceNaCT cRNA by measuring the uptake of [^{14}C]citrate (40 μM) at pH 6.5. The uptake in cRNA injected oocytes was 12.9 ± 0.8 pmol/oocyte/15 min. This value was about 45-fold higher than the uptake measured under identical conditions in oocytes injected with water (0.3 ± 0.01 pmol/oocyte/15 min) (Fig. 24A). Next, the electrogenic nature of the transport process was examined.

Perfusion of the ceNaCT cRNA-injected oocytes with citrate (250 μM) in the presence of Na^+ (100 mM NaCl) induced inward currents, detectable by the TEVC method at a holding membrane potential of -50 mV (Fig. 24B). Perfusion of the oocytes with succinate also induced similar inward currents, though the magnitude of the currents was less than that induced by citrate. These data show unequivocally that ceNaCT-mediated transport process is electrogenic irrespective of whether the transported substrate is a dicarboxylate or a tricarboxylate. The citrate- and succinate-induced currents were obligatorily dependent on the presence of Na^+ . In the absence of Na^+ (NaCl was substituted by choline chloride), perfusion of the oocytes with citrate did not induce any detectable current (Fig. 24B). On the other hand, when chloride in the perfusion buffer was replaced with gluconate, the citrate-induced current remained unaltered. These data show that the transport process mediated by ceNaCT is Na^+ dependent and that Cl^- ions do not have any role in the transport process. Similar results were obtained with succinate in terms of Na^+ -

dependence and Cl^- -independence. The substrate (citrate or succinate)-induced inward currents in ceNaCT cRNA-injected oocytes were influenced by the pH in the perfusion buffer (Fig. 24C). The magnitude of the currents was increased significantly when the pH of the perfusion buffer was changed from 7.5 to 6.5. The same was observed when the buffer pH was changed from 5.5 to 6.5.

The citrate-induced currents of ceNaCT were further analyzed in terms of their dependence on membrane potential. Steady-state currents induced by citrate over a concentration range of 10 μM to 1 mM were measured at different testing membrane potentials (-50 mV to -150 mV). At each of these testing membrane potentials, the citrate-induced currents were saturable with respect to citrate concentration (Fig. 24D). The maximal current induced by citrate however increased with increasing testing membrane potential. The data show that the transport rate increases with increasing membrane potential, suggesting a role for membrane potential as a driving force for the transport process. Thus, the transport process mediated by ceNaCT derives its driving force from the electrochemical Na^+ gradient. The relationship between the substrate concentration and the induced current was hyperbolic at each of the testing membrane potentials. The data were analyzed at each of the testing membrane potentials according to the Michaelis-Menten equation. The Michaelis-Menten constant ($K_{0.5}$), the concentration of citrate needed for the induction of half-maximal current, was $34 \pm 8 \mu\text{M}$ at a testing membrane potential of -70 mV. This value did not change significantly with different testing membrane potentials. However, the maximal current (I_{max}) increased with increasing testing membrane potential. The value for I_{max} was $12.3 \pm 0.6 \text{ nA}$ at a testing membrane potential of -50 mV and this value increased gradually to $61.1 \pm 2.6 \text{ nA}$ as the testing membrane potential increased to -150 mV. These data show that the membrane potential does not influence the substrate affinity of the transporter but it enhances the maximal velocity of the transport process.

Developmental stage-specific expression pattern of the *nact* gene. To monitor the relative expression levels of NaCT mRNA during different stages of *C. elegans* development, synchronized cultures were obtained and total RNA was isolated at each of the following four stages of development: embryo, early

larva (larva stages 1 and 2), late larva (larva stages 3 and 4), and adult. The steady-state levels of mRNA for NaCT were then determined by semi-quantitative RT-PCR with 18S rRNA as an internal control to adjust for variations in RNA input into RT-PCR reactions. The levels of NaCT mRNA were compared at different developmental stages based on relative intensities of the NaCT-specific RT-PCR product. The *nact* gene was expressed at much higher levels during the early embryo stage than during the adult stage. However, the expression was detectable all through the different stages of development. The levels of NaCT mRNA as assessed by the relative band intensities of RT-PCR products for NaCT and 18S rRNA at the stages of embryo, early larva, late larva, and adult were 5.6, 2.1, 0.8, and 1.4.

Tissue-specific expression pattern of the *nact* gene. Tissue expression pattern of the *nact* gene in *C. elegans* was investigated using the transgenic GFP fusion technique in which the transgene consisted of the *nact* gene-specific promoter fused with GFP cDNA. The expression of GFP in this fusion gene is controlled by the *nact* gene-specific promoter. Therefore, the expression pattern of GFP in transgenic *C. elegans* expressing the fusion gene would match the expression pattern of the native *nact* gene. With this technique, it was found that GFP expression is restricted to the intestinal tract in this organism. This expression pattern is evident from the early larva stage through the adult stage. The GFP fluorescence is detectable throughout the intestinal tract, starting from the pharynx all the way through the anus. The expression level of GFP is significantly greater in the anterior half of the intestine than in the posterior half. This expression pattern was confirmed with at least 10 transgenic animals.

Influence of RNAi-mediated knockdown of the function of NaCT on life span, body size and fat deposit. Knockdown of the function of NaCT by feeding wild type N2 worms with bacteria expressing the ceNaCT-specific dsRNA caused a significant increase in average life span and maximal life span of the organism (Fig. 25A). Average life span of the worms fed on bacteria harboring ceNaCT-specific dsRNA was 19.4 ± 0.3 days (N=211); average life span of the worms fed on bacteria harboring the empty vector pPD129 was the same as that of wild-type N2 worms (16.3 ± 0.2 days; N=180). The increase in

average life span induced by ceNaCT knockdown was 19% ($p < 0.001$). The DAF-2 knockdown was also included as a positive control in these experiments. Worms fed on bacteria expressing DAF-2-specific dsRNA exhibited an average life span of 31.4 ± 0.6 days ($N=60$), showing that knockdown of the function of DAF-2 doubles average life span. This influence of DAF-2 knockdown on life span is similar to the influence of homozygous knockout of *daf-2* gene function on life span ((Kenyon et al., *Nature* (1993); 366: 461-464, Wolkow et al., *Science* (2000); 290:147-150). A “lean” phenotype was detected when the *cenact* gene was knocked down by bacteria-mediated RNAi approach. Body length and body width were smaller in ceNaCT-RNAi worms than those of control worms. Therefore, the calculated body size was significantly reduced (approximately 40%) in ceNaCT-RNAi worms (2.95 ± 0.13 nl, $N=60$) in comparison with the control vector fed worms (4.91 ± 0.05 nl, $N=58$, Fig. 25B). This interesting phenotype was not observed when ceNaDC1 or ceNaDC2 was knocked down by a similar experimental approach (ceNaDC1 knockdown: 4.92 ± 0.07 nl, $N=45$; ceNaDC2 knockdown: 5.05 ± 0.07 nl, $N=53$). The intestinal fat content was also analyzed by a laser confocal fluorescence microscopy approach using Nile red (5H-benzo[α]phenoxazine-5-one, 9-diethylamino) to stain intracellular lipid droplets in live *C. elegans* under the influence of the ceNaCT gene-specific RNAi (Ashrafi et al., *Nature* (2003); 421: 268-272). The intensity of Nile red staining in this organism was reduced markedly to 56% of the control value when the NaCT was knocked down by RNAi ($P < 0.001$, $N=13$; Fig. 26). In these experiments, the control worms ($N=8$) were fed on bacteria harboring empty vector pPD129. Under the same experimental conditions, the intensity of Nile red staining was not altered when NaDC1 was knocked down by RNA.

Interestingly, the knockdown of NaDC2 caused a significant decrease in the intensity of Nile red staining, but the decrease was much less than that seen with NaCT knockdown. In the case of NaDC2 knockdown, the decrease in fat content was 30% ($P < 0.05$) (Fig. 8D). These experiments included a group of worms in which DAF-2 was knocked down as a positive control because it has been shown that knockdown of this gene function leads to a significant increase

in body fat storage (Ashrafi et al., *Nature* (2003);421: 268-272). As expected, the knockdown of DAF-2 led to a 12% increase ($P < 0.05$) in the intensity of Nile red staining (Fig. 26).

5 DISCUSSION

This example describes the cloning and functional characterization of a Na^+ -coupled citrate transporter (NaCT) in *C. elegans*. The *C. elegans* NaCT transports the tricarboxylate citrate as well as several dicarboxylates such as succinate and α -ketoglutarate. The pH-dependence of the transport of citrate and succinate via this transporter shows that the trivalent form of citrate and the divalent form of succinate are the transportable substrates at physiological pH. The transport process is Na^+ -dependent, Cl^- -independent, and electrogenic for both substrates. Functionally, the *C. elegans* NaCT is similar to rat NaCT (Example 2) and human NaCT (Example 3). The *C. elegans* NaCT and the mammalian NaCT are structurally similar to *Drosophila* Indy (Example 1). NaCTs are also similar to *Drosophila* Indy with respect to the ability of these transporters to recognize citrate as a substrate. However, *Drosophila* Indy is a Na^+ -independent citrate transporter. The transport characteristics of *C. elegans* NaCT are distinct from those of NaDC1 and NaDC2, previously identified in this organism. The two NaDCs transport succinate and other dicarboxylates much more effectively than citrate. Thus, the two NaDCs in *C. elegans* correspond to mammalian NaDC1 and NaDC3, whereas the transporter reported in this example corresponds to mammalian NaCT.

The unique functional feature of *C. elegans* NaCT is its ability to transport the tricarboxylate citrate as well as the dicarboxylates such as succinate. Mammalian NaDCs (NaDC1 and NaDC3) are able to transport only dicarboxylates. Even though these transporters can transport citrate, it is only the dianionic form of citrate that is recognized as the substrate. Therefore, mammalian NaDCs are truly Na^+ -coupled dicarboxylate transporters. In contrast, NaCT possess the ability to transport dicarboxylates as well as the tricarboxylate citrate. The pH-dependence of succinate uptake and citrate uptake mediated by *C. elegans* NaCT shows that the transporter recognizes succinate in its divalent form and citrate in its trivalent form as the substrates.

This example also provides information on the electrophysiological characteristics of *C. elegans* NaCT, as an electrogenic transporter. The transport process involves transfer of positive charge across the membrane irrespective of whether the transported substrate is a tricarboxylate or a dicarboxylate. Since the transport of citrate, a tricarboxylate, is an electrogenic process, there should be at least 4 Na⁺ ions involved in the transport process. The predicted Na⁺:substrate stoichiometry of 4:1 makes NaCT a very efficient concentrative transporter. In addition to the chemical Na⁺ gradient, the membrane potential also serves as a driving force for this transporter. The substrate affinity of the transporter is not influenced by membrane potential, but the maximal velocity of the transport process is enhanced markedly by hyperpolarization.

The extracellular pH influences markedly the transport of citrate via *C. elegans* NaCT. When the pH was changed from 7.5 to 6.5, the transport rate increased about 3-fold. At pH 7.5, only 7% of citrate exists in its divalent form. The concentration of the divalent form increases six fold to 44% when the pH is changed to 6.5. This pH-dependence of the transport function of *C. elegans* NaCT reflects the influence of pH on the translocation process as well as on the influence of pH on the ionization of the substrates. The pH-dependent stimulation of citrate uptake, when the pH was changes from 7.5 to 6.5, was about 3-fold in the mammalian cell expression system whereas the corresponding value was about 1.25-fold in the *X. laevis* oocyte expression system. This difference was most likely due to different citrate concentrations used (10 μM in the case of mammalian cells and 250 μM in the case of oocytes). The concentrations of the trivalent form of citrate relative to the K_t value were much lower in experiments with mammalian cells than with oocytes.

Irrespective of the charge of the transported substrate, the transport process mediated by *C. elegans* NaCT is electrogenic, with at least 4 Na⁺ ions involved in the transport process. If the Na⁺:substrate stoichiometry is 4:1 irrespective of the charge nature of the substrate, the net transfer of charge across the membrane is expected to differ depending on whether the transported substrate is a tricarboxylate or a dicarboxylate. The transport of citrate, a tricarboxylate, would result in the transfer of only one positive charge whereas

the transport of dicarboxylates such as succinate would result in the transfer of two positive charges. The amount of charge that is transferred across the membrane during the transport process can be quantified electrophysiologically in *X. laevis* oocytes by measuring the charge transfer and the substrate transfer simultaneously. The magnitude of currents induced by succinate and citrate in oocytes expressing rat NaCT is at least 10 times higher than that seen in oocytes expressing *C. elegans* NaCT. This made the analysis of the charge: substrate transfer ratio possible in the case of rat NaCT using the Fetchex protocol. These studies have shown that the charge: substrate transfer ratio is 1:1 for citrate and 2:1 for succinate.

Since citrate is an excellent substrate for NaCT, the physiological function of this transporter will be related to energy production and synthesis of fatty acids and cholesterol. The successful cloning of this transporter from *C. elegans* has provided an opportunity to demonstrate this relationship. Studies of NaCT knockdown using the RNAi technique in *C. elegans* show suppression of NaCT function does lead to a significant increase in the average life span of the organism. Interestingly, previous studies have shown that suppression of NaDC2 function also leads to life span extension. This is not surprising because both NaCT and NaDC2 are functionally similar in terms of transport of succinate and other dicarboxylate intermediates of TCA cycle. Therefore, knockdown of NaCT or NaDC2 impair the utilization of succinate and other TCA cycle intermediates for energy production, leading to a metabolic state similar to that of caloric restriction and hence to life span extension. However, even though NaCT and NaDC2 are similar in their ability to transport succinate and other dicarboxylates, they differ markedly in their ability to transport citrate. This tricarboxylate occupies a unique position in metabolism in that it is not only an intermediate in the TCA cycle but also a precursor for the synthesis of fatty acids and cholesterol.

Therefore, these two transporters differ in their role in body fat storage. The knockdown of NaCT function leads to a marked decrease (65%) in the fat content of the organism. Similar maneuver with NaDC2 also decreases the fat content, but to a much lesser degree (30%). This is because succinate and other dicarboxylate intermediates of the TCA cycle can enter the TCA cycle leading

to the conversion of their carbon skeleton to citrate within the mitochondria. The mitochondria-derived citrate can subsequently function as a precursor for fatty acid and cholesterol synthesis in the cytoplasm after being transported out of the mitochondria. Since the influence of NaCT knockdown on body fat content is much higher than that of NaDC2 knockdown, the utilization of extracellular citrate is quantitatively more important than the mitochondria-derived citrate in the biosynthesis of fatty acids. NaDC1, also transports succinate and other dicarboxylate intermediates of the TCA cycle as does NaDC2, but the knockdown of NaDC1 has no detectable influence on body fat storage and on life span. This may be because NaDC1 is a low affinity transporter whereas NaDC2 is a high affinity transporter.

In summary, in this example, a Na^+ -coupled transporter was identified in *C. elegans* which is an ortholog of mammalian NaCT. Similar to the mammalian NaCT, *C. elegans* NaCT transports not only succinate and other dicarboxylate intermediates of the TCA cycle but also the tricarboxylate citrate. This example demonstrates the role of this novel transporter in metabolic functions in *C. elegans*. This transporter plays a critical role in life span and body fat storage. The knockdown of the function of the transporter in this organism leads to an increase in life span and a decrease in body fat content.

Example 5

Murine Na^+ -coupled Citrate Transporter (NaCT):

Primary Structure, Genomic Organization, and Transport Function

This example presents the molecular cloning and functional characterization of mouse NaCT, the murine ortholog of *Drosophila* Indy, and provides evidence for the electrogenic nature of the transport process mediated by the rodent NaCT, irrespective of whether the transported substrate is a tricarboxylate or a dicarboxylate. Mouse NaCT consists of 572 amino acids and is highly similar to rat and human NaCTs in primary sequence. The murine *nact* gene coding for the transporter is approximately 23 kb-long and consists of

12 exons. When expressed in mammalian cells, the cloned transporter mediates the Na⁺-coupled transport of citrate and succinate. Competition experiments reveal that mouse NaCT also recognizes other citric acid cycle intermediates such as malate, fumarate, and 2-oxo-glutarate as excellent substrates. The
5 Michaelis-Menten constant for the transport process is $22 \pm 2 \mu\text{M}$ for citrate and $33 \pm 4 \mu\text{M}$ for succinate.

The transport process is obligatorily dependent on Na⁺ and the Na⁺-activation kinetics indicates that multiple Na⁺ ions are involved in the activation process. Extracellular pH has a differential effect on the transport function of
10 mouse NaCT depending on whether the transported substrate is citrate or succinate. When examined in the *Xenopus laevis* oocyte expression system with the two-microelectrode voltage-clamp technique, the transport process mediated by mouse NaCT is electrogenic. The charge-to-substrate ratio is 1 for citrate and 2 for succinate. The most likely transport mechanism predicted by these studies
15 involves the transport of citrate as a trivalent anion and succinate as a divalent anion with a fixed Na⁺:substrate stoichiometry of 4:1. This example provides the first unequivocal evidence for the electrogenic nature of mammalian NaCT, providing the first direct evidence that NaCT can transport its substrates in trivalent as well as divalent forms with a fixed Na⁺:substrate stoichiometry of
20 4:1.

MATERIALS AND METHODS

Cloning of mouse NaCT cDNA. A search of GenBank database using the rat and human NaCT amino acid sequences as queries identified a sequence
25 (Genbank Accession No. XM_137672), predicted from the NCBI (National Center for Biotechnology Information) contig NT_039520 by automated computational analysis using the gene prediction method GenomeScan, which is most likely the mouse ortholog of NaCT. This 1947 bp-long (open reading frame plus the termination codon) sequence codes for a putative protein
30 consisting of 648 amino acids. This putative protein is 76 amino acids longer than the cloned rat NaCT and 80 amino acids longer than the cloned human NaCT. Since this is a theoretically predicted sequence of the coding region by computational analysis, it does not necessarily represent the sequence of the

actual mRNA. A comparison of the nucleotide sequence of this putative mRNA with that of the mouse gene (contig NT_039520) led to the identification of the exons that code for the sequences containing the translational start codon and the translational termination codon.

5 In addition, a search of GenBank database revealed that the sequence containing the translational termination codon is located in two established sequence tags (ESTs) (gi10645928 and gi6101350). This information allowed the design of primers suitable for amplification of the entire coding region of mouse NaCT using mouse brain mRNA as the template. The upstream primer, 10 containing the translational start codon (shown in bold), was 5'-GTCTCCCTTTCACGCGATGG-3' (SEQ ID NO:27) and the downstream primer, located just two nucleotides downstream of the translational termination codon, was 5'-TCGTCTAGAGCCTTGTGCTCTTGCGGCTCT-3' (SEQ ID NO:28). The underlined sequence in the downstream primer is an *Xba*I site, 15 added to the 5'-end of the primer for cloning purpose. RT-PCR with these primers and mouse brain mRNA as the template yielded an approximately 1.8 kb product. This product was subcloned into pGEM-T Easy vector. The insert was then released from the plasmid by digestion with *Eco*RI and *Xba*I and subcloned into the vector pGH19 at the *Eco*RI/*Xba*I site. The pGH19 vector 20 contains the 3'-untranslated region of the *Xenopus* β -globin gene downstream of the cloning site and has been shown to increase the expression levels of heterologous genes in oocytes (Liman et al., *Neuron* (1992);9: 861-871, Trudeau et al., *Science* (1995);269: 92-95). This vector also contains the T7 promoter upstream of the cloning site and thus is suitable for functional 25 expression in mammalian cells using the vaccinia virus expression technique (Inoue et al., *J. Biol. Chem.* (2002);277: 39469-39476 and Inoue et al., *Biochem. Biophys. Res. Commun.* (2002);299: 465-471). The sense, as well as antisense, strands of the cDNA insert were sequenced by the *Taq* DyeDeoxy terminator cycle method using an automated Perkin-Elmer Applied Biosystems 377 Prism 30 DNA sequencer. The sequence was analyzed using the NCBI server, available on the worldwide web at ncbi.nlm.nih.gov.

Functional expression of mouse NaCT cDNA in human retinal pigment epithelial (HRPE) cells. The cloned mouse NaCT cDNA was expressed

functionally in HRPE cells using the vaccinia virus expression technique (Inoue et al., *J. Biol. Chem.* (2002);277: 39469-39476 and Inoue et al., *Biochem.*

Biophys. Res. Commun. (2002);299: 465-471). Cells transfected with vector alone served as control for determination of endogenous transport activities.

- 5 The transport of [^{14}C]-citrate (sp. radioactivity, 55 mCi/mmol) and [^3H]-succinate (sp. radioactivity, 40 Ci/mmol), both from Moravek Biochemicals (Brea, CA, USA), was measured in control cells and in cDNA-transfected cells in parallel. Based on time course studies, initial transport rates for citrate and succinate were measured using a 30-min incubation and a 15-min incubation, respectively. The transport buffer was 25 mM Hepes/Tris (pH 7.5) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 5 mM glucose.

- Interaction of various citric acid cycle intermediates and related compounds with the transporter was assessed by monitoring the ability of these compounds to compete with citrate and succinate for NaCT-mediated transport.
- 15 The NaCT-specific transport was determined by subtracting the transport values measured in vector-transfected cells from the transport values measured in cDNA-transfected cells. Substrate saturation kinetics was analyzed by fitting the NaCT-specific transport data to the Michaelis-Menten equation. The kinetic constants (Michaelis-Menten constant, K_t and maximal velocity, V_{max}) were calculated by using non-linear as well as linear regression methods. The dependence of NaCT-mediated transport of citrate and succinate on Na^+ was determined by comparing the transport values measured in the presence of varying concentrations of Na^+ where NaCl was replaced isoosmotically with *N*-methyl-D-glucamine chloride.
- 20 The Na^+ -activation kinetics was analyzed by fitting the NaCT-specific transport data to the Hill equation. The Hill coefficient (n_H , the number of Na^+ involved in the activation process) was determined by using non-linear as well as linear regression methods.

- Functional expression of mouse and rat NaCTs in *Xenopus laevis* oocytes. Capped cRNA from the cloned mouse NaCT cDNA was synthesized using the mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX, USA). In some experiments, rat NaCT of Example 2 was used for functional expression. Mature oocytes from *Xenopus laevis* were isolated by treatment with
- 30

collagenase A (1.6 mg/ml), manually defolliculated, and maintained at 18° C in modified Barth's medium supplemented with 10 mg/ml gentamycin as described previously (Hatanaka et al., *J. Clin. Invest.* (2001);107: 1035-1043 and Nakanishi et al., *J. Physiol.* (2001);532: 297-304). On the following day, oocytes were injected with 50 ng cRNA. Water-injected oocytes served as controls. The oocytes were used for electrophysiological studies 4-6 days after cRNA injection. Electrophysiological studies were performed by the two-microelectrode voltage-clamp method (Hatanaka et al., *J. Clin. Invest.* (2001);107: 1035-1043 and Nakanishi et al., *J. Physiol.* (2001);532: 297-304). Oocytes were perfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 3 mM Hepes, 3 mM Mes, and 3 mM Tris, pH 7.5), followed by the same buffer containing citrate and succinate. The membrane potential was clamped at - 50 mV. The charge-to-substrate ratio was determined for citrate and succinate in three different oocytes. The oocytes were perfused with 50 µM [¹⁴C]-citrate or 50 µM succinate (unlabeled plus radiolabeled succinate) and inward currents were monitored over a period of 10 min. At the end of the experiment, the amount of citrate and succinate transported into the oocytes was calculated by measuring the radioactivity associated with the oocytes. The area within the curve describing the relationship between the time and inward current was integrated to calculate the charge transferred into the oocyte during incubation with citrate or succinate. The values for substrate transport and charge transfer were used to determine the charge-to-substrate ratio.

Data analysis. Experiments with HRPE cells were repeated three times with three independent transfections and transport measurements were made in duplicate in each experiment. Electrophysiological measurements of substrate-induced currents were repeated at least three times with separate oocytes. The data are presented as means ± S. E. of these replicates. The kinetic parameters were calculated using the commercially available computer program Sigma Plot, version 6.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Structural features of mouse NaCT. The coding region of mouse NaCT cDNA (SEQ ID NO:9) is 1719 bp long (including the termination codon) and the predicted protein coded by this cDNA consists of 572 amino acids (SEQ ID NO:10). See Fig. 27. This protein is 76 amino acids shorter than the protein predicted by computational analysis (GenBank Accession No. XM_137672). A comparison of the nucleotide sequence of this predicted mRNA with that of the mouse *nact* gene located in the contig NT_039520 shows that the extra 76 amino acids arise from intron 1 using alternative splice junctions as predicted by computational analysis. This sequence is however not found in the mRNA isolated from mouse brain. The mouse NaCT protein cloned from the brain shows 86% sequence identity (93% similarity) with rat NaCT and 74% sequence identity (85% similarity) with human NaCT (Fig. 28A). The amino acid sequence identity is much lower with other members of the gene family (SLC13), namely the low-affinity Na⁺-coupled dicarboxylate transporter NaDC1 (mouse NaCT versus mouse NaDC1, 50% identity), the high-affinity Na⁺-coupled dicarboxylate transporter NaDC3 (mouse NaCT versus mouse NaDC3, 44% identity), the Na⁺-coupled sulfate transporter NaSi1 (mouse NaCT versus mouse NaSi1, 40% identity) and the sulfate transporter SUT1 (mouse NaCT versus mouse SUT1, 39% identity).

NaCT represents the mammalian ortholog of *Drosophila* Indy with which the cloned mouse NaCT shares 33% identity and 57% similarity in amino acid sequence. Hydropathy analysis, according to the computer program Tmpred, available on the worldwide web at ch.embnet.org/cgi-bin/TMPRED_form_parser, predicts two alternative models for mouse NaCT. According to the strongly preferred model, mouse NaCT possesses 13 transmembrane domains with its amino terminus facing the cytoplasmic side of the membrane and C-terminus facing the exoplasmic side of the membrane. The alternative model predicts mouse NaCT as possessing 12 transmembrane domains with its N-terminus and C-terminus facing the exoplasmic side of the membrane. There are three putative *N*-glycosylation sites, Asn-179, Asn-382, and Asn-566. Of these, only Asn-566 is preserved in rat and human NaCT. In the topology model with 13 transmembrane domains, Asn-179 and Asn-382 are

located in the cytoplasmic loops and Asn-566 lies on the exoplasmic side. In the topology model with 12 transmembrane domains, all three Asn residues are located on the exoplasmic side of the membrane.

Exon-intron organization of mouse *nact* gene. The mouse gene coding for NaCT is located on chromosome 11 and consists of 12 exons (Fig. 28B). Exon 1 codes for the translational start site and exon 12 codes for the translational termination site. The size of the gene is approximately 23 kb, excluding the promoter region. The exact lengths of individual exons and introns and the identity of the splice junctions are described in Table 9.

Functional features of mouse NaCT as assessed in a mammalian cell expression system. Fig. 29 shows the transport of succinate and citrate by mouse NaCT when expressed heterologously in HRPE cells. The transport of succinate (50 nM) is 7.8-fold higher in cells transfected with mouse NaCT cDNA than in cells transfected with vector alone (42.7 ± 4.6 fmol/ 10^6 cells/min in cDNA-transfected cells and 5.5 ± 0.7 fmol/ 10^6 cells/min in vector-transfected cells). The transport of citrate (20 μ M) is 20.2-fold higher in cells transfected with mouse NaCT cDNA than in cells transfected with vector alone (48.4 ± 1.0 pmol/ 10^6 cells/min in cDNA-transfected cells and 2.4 ± 0.1 pmol/ 10^6 cells/min in vector-transfected cells). These data show that mouse NaCT is able to transport succinate as well as citrate and that the magnitude of citrate transport is much higher than that of succinate transport in terms of stimulation of transport induced by the cloned transporter. Kinetic analysis indicates that the transport of citrate and succinate mediated by mouse NaCT is saturable (Fig. 30). The Michaelis-Menten constant (K_t) is 22 ± 2 μ M for citrate and 33 ± 4 μ M for succinate. The corresponding values for the maximal velocity (V_{max}) are 118 ± 6 pmol/ 10^6 cells/min (citrate) and 80 ± 5 pmol/ 10^6 cells/min (succinate), respectively.

The transport of citrate and succinate via mouse NaCT is obligatorily dependent on the presence of Na^+ . Fig. 31 describes the Na^+ -activation kinetics for the transport of citrate and succinate. The relationship between the transport rate and Na^+ concentration is sigmoidal for both substrates. Analysis of the data according to Hill equation shows that the Hill coefficient (n_H ; the number of Na^+ involved in the activation process) is 3.3 ± 0.6 for citrate and 2.0 ± 0.3 for

succinate. Interestingly, the transport of citrate reaches saturation with a Na^+ concentration of 120 mM and therefore the $K_{0.5}$ for Na^+ (i.e., the concentration of Na^+ needed for half-maximal activation of the transport process) could be determined without ambiguity. The value is 72 ± 6 mM. In contrast, the
5 transport of succinate does not reach saturation within the concentration range of Na^+ tested (upto 140 mM) and therefore the $K_{0.5}$ could not be reliably determined.

The substrate specificity of mouse NaCT was investigated by assessing the ability of various citric acid cycle intermediates and related compounds to
10 compete with [^{14}C]-citrate and [^3H]-succinate for transport via mouse NaCT. These studies have shown that citrate, succinate, fumarate, malate, and 2-oxo-glutarate are recognized by the transporter as substrates. These five compounds effectively competed with radiolabeled citrate and succinate for transport via mouse NaCT (Table 10). The monocarboxylates pyruvate and lactate exhibit no
15 or little inhibitory activity for the transport of citrate and succinate. Similarly, maleate, the *cis* isomer of fumarate, is also not effective as an inhibitor. When compared with citrate, the tricarboxylates isocitrate and *cis*-aconitate also do not interact with the transporter effectively.

Citrate is a tricarboxylate with pK values of 3.1, 4.8, and 6.4. Succinate
20 is a dicarboxylate with pK values of 4.2 and 5.6. At pH 7.5, citrate exists about 90% as a trivalent anion and about 10% as a divalent anion. Succinate, on the other hand, exists almost completely as a divalent anion at pH 7.5. Therefore, it would be of interest to compare the influence of pH on the NaCT-mediated transport of citrate and succinate under identical conditions. The data, shown in
25 Fig. 32, indicate that pH has differential influence on the transport of citrate (10 μM) and succinate (10 μM) via mouse NaCT. The transport of citrate exhibits a clear optimum pH at 7. The transport rate decreases significantly when the pH is made more alkaline or more acidic than 7. In contrast, the transport of succinate is maximal in the pH range 7.5-8.5 and decreases significantly when
30 the pH is less than 7.5.

Functional features of mouse NaCT as assessed in the *Xenopus laevis* oocyte expression system. The studies of Examples 2 and 3 have indicated that mammalian NaCT is electrogenic, based on the influence of membrane

depolarization of the transport activity. The present studies with mouse NaCT show that both succinate and citrate are transported via this transporter. Even though the data from the Na^+ -activation kinetics have shown that multiple Na^+ ions are involved in the transport process, the participation of 2 Na^+ ions in the activation of succinate transport and 3 Na^+ ions in the activation of citrate transport does not necessarily predict the electrogenic nature of these transport processes. Therefore, the *Xenopus* oocyte expression system was chosen to analyze the electrogenicity of mouse NaCT.

Expression of mouse NaCT in oocytes led to a marked increase in the uptake of [^{14}C]-citrate and [^3H]-succinate, indicating functional expression of the transporter. These oocytes were then used for electrophysiological studies to determine if the perfusion of the oocytes in the presence of substrates leads to inward currents when monitored by the two-microelectrode voltage-clamp method. These studies have shown that exposure of the NaCT-expressing oocytes to citrate induces measurable inward currents (30 ± 7 nA at 0.5 mM citrate in three different oocytes) (Fig. 33). The citrate-induced current is obligatorily dependent on the presence of Na^+ . Isoosmotic replacement of Na^+ with *N*-methyl-D-glucamine abolishes the citrate-induced currents almost completely. These currents are not dependent on Cl^- as isoosmotic replacement of Cl^- with gluconate does not affect the citrate-induced currents (Fig. 33). Exposure of the oocytes to succinate also induces inward currents in a Na^+ -dependent manner.

An analysis of the charge-to-substrate ratio for the transport process was motivated by the findings that the transport of citrate as well as succinate occurs via an electrogenic process. This raises three different possibilities in terms of transport mechanism. First, both citrate and succinate are transported as divalent anions and the Na^+ :substrate stoichiometry is 3:1. In this case, the charge-to-substrate ratio would be 1 for both substrates. Second, citrate is transported as a trivalent anion and succinate is transported as a divalent anion, but the Na^+ :substrate stoichiometry changes from 4:1 for citrate to 3:1 for succinate. In this case also, the charge-to-substrate ratio would remain as 1. Third, citrate is transported as a trivalent anion and succinate is transported as a divalent anion, and the Na^+ :substrate stoichiometry remains as 4:1 for both

substrates. In this case, the charge-to-substrate ratio would vary depending on the substrate. This ratio would be 1 for citrate but 2 for succinate.

To differentiate among these three possibilities, the FetchEx method, in which the NaCT-expressing oocytes are perfused with radiolabeled citrate or succinate for a given time period in which the substrate-induced inward currents are monitored, was employed. At the end of the perfusion period, the amount of citrate or succinate transported into the oocytes is measured. The amount of charge transferred into the same oocyte can be calculated from the inward currents. This would allow the determination of the charge-to-substrate ratio. However, this requires a robust electrogenicity of the transport process so that radiolabeled substrate can be mixed with adequate amounts of unlabeled substrate to allow measurable inward currents for charge transfer calculations and, at the same time, to allow measurable transfer of radiolabel into the oocyte so that the amount of substrate transferred can be measured. Previous experience (Wang et al., *Am. J. Physiol.* (2000);278: C1019-C1030 and Fei et al., *Biochim. Biophys. Acta* (1999);1418: 344-351), has shown that the substrate-induced currents via mouse NaCT are not sufficient to determine the charge-to-substrate ratio using our electrophysiological set up.

Therefore rat NaCT was used, to see if this transporter is associated with a higher magnitude of substrate-induced currents compared to mouse NaCT. This indeed turned out to be the case. Perfusion of the oocytes, which expressed rat NaCT, to 0.5 mM citrate was found to induce 3-fold higher currents compared to mouse NaCT (Fig. 33). The ability of rat NaCT to transport other intermediates of the citric acid cycle was determined, by comparing the magnitude of inward currents induced by these compounds (Fig. 34). When the concentration of the substrate was kept constant at 0.5 mM, citrate induced the maximum current compared to various putative substrates tested. Fumarate, succinate, malate, and 2-oxo-glutarate, the dicarboxylate intermediates of the citric acid cycle, also induced appreciable currents, but the magnitude of the currents was significantly less than that induced by citrate (20-60% of citrate-induced currents). *Cis*-aconitate induced currents that amounted to approximately 10% of citrate-induced currents. Isocitrate, pyruvate, and lactate induced no or little currents. Since rat NaCT induced marked currents

with citrate as well as succinate, the charge-to-substrate ratio for the transport process using rat NaCT rather than mouse NaCT was analyzed. Fig. 35 describes the data for the transport of citrate and succinate via rat NaCT. Even though the transport of both substrates via the transporter is electrogenic, the quantity of the charge transferred into the oocytes per a given amount of the substrate transfer is relatively higher for succinate than for citrate. The charge-to-substrate transfer ratio is 2 for succinate whereas the corresponding value is 1 for citrate, indicating that the Na^+ :substrate stoichiometry is 4:1 irrespective of whether transported substrate is citrate or succinate.

DISCUSSION

The amino acid sequence of mouse NaCT cloned from the brain differs from that predicted from the mouse gene sequence by computational analysis by a stretch of 76 amino acids that is predicted by the GenomeScan but not found in the cloned NaCT. This additional sequence arises from the use of alternative splice junctions predicted by the GenomeScan in intron 1. The nucleotide sequence of the cloned NaCT cDNA (SEQ ID NO:9) shows that exon 1 is 102 bp-long (starting from the translational initiation codon ATG) and that exon 2 is 129 bp-long. However, according to the GenomeScan prediction, exon 1 contains an additional 194 bp sequence at its 3'-end and exon 2 contains an additional 34 bp sequence at its 5'-end. This stretch of 228 bp, predicted to be a part of the coding region, gives rise to the extra 76 amino acids. This sequence is however not found in the cDNA cloned from mouse brain. Whether these alternative splice junctions are used in tissues other than the brain is not known. NaCT has been cloned from rat brain and from a human liver cell line, but there is no evidence for the presence of alternative splice variants in these tissues. Therefore, it seems very unlikely that the stretch of the additional 76 amino acids predicted by the GenomeScan is actually found in NaCT expressed in any tissue.

Analysis of the amino acid sequence (SEQ ID NO:10) of the cloned mouse NaCT using the TMpred program predicts two different topology models, one with 13 transmembrane domains with its N-terminus placed on the cytoplasmic side of the membrane and C-terminus placed on the external

surface of the membrane and the other with 12 transmembrane domains with its N-terminus and C-terminus placed on the external surface. This protein also possesses three putative *N*-glycosylation sites, of which Asn-566 is conserved in rat and human NaCTs. Additional evidence that the corresponding Asn residue in human NaCT (Asn-562) is most likely to be *N*-glycosylated is that treatment of HepG2 cells with tunicamycin, an inhibitor of *N*-linked glycosylation, leads to a significant inhibition of NaCT activity. These data suggest that Asn-566 in mouse NaCT is also likely to be *N*-glycosylated. Both topology models with either 12 or 13 transmembrane domains are in agreement with the potential *N*-glycosylation of Asn-566 because in both models this residue is located on the exoplasmic side of the membrane. Interestingly, analysis of the amino acid sequences of rat and human NaCTs using the same TMPred program indicates that these proteins possess 12 transmembrane domains instead of 13. However, the program indicates that both N-terminus and C-terminus are most likely to be located on the external surface of the membrane, and places the conserved *N*-glycosylation site in the C-terminus tail in rat and human NaCTs on the external side of the membrane.

While Example 2 and Example 3 present the cloning and functional characteristics of rat and human NaCT, the present example provides important information, for the first time, on the transport mechanism of NaCT with respect to its Na^+ :substrate stoichiometry and electrogenicity. Mouse NaCT, when expressed in mammalian cells, transports not only citrate but also succinate. This is similar to rat NaCT, as shown in Example 2. In contrast, human NaCT exhibits very little ability to transport succinate (see Example 3). pH-dependence of the transport process mediated by mouse NaCT shows that the transport of succinate remains almost unaffected in the pH range 7.0-8.5 whereas the transport of citrate increases markedly when the pH is changed from 8.5 to 7.0. These data can be taken as evidence that citrate is recognized by NaCT in its protonated divalent anionic form because the fraction of the divalent form of citrate increases as the pH is changed from 8.5 to 7.0. The divalent form of succinate does not change significantly in the pH range 7.0-8.5.

Similar conclusions have been drawn in the case of NaDC1 and NaDC3 (Pajor, *Annu. Rev. Physiol.* (1999);61: 663-682 and Pajor, *J. Membr. Biol.*

(2000);175: 1-8). Na^+ -activation kinetics of mouse NaCT shows that multiple Na^+ ions are involved in the transport process. The Na^+ :substrate stoichiometry is 3:1 for citrate and 2:1 for succinate. These data, together with the results from the pH-dependence studies, would suggest that citrate transport is electrogenic whereas succinate transport is electroneutral. This however is not the case as evident from the oocyte expression system. In contrast to the studies with the mammalian cell expression system, electrophysiological studies with the oocyte expression system provide evidence for a completely different transport mechanism for mouse NaCT. The transport of citrate as well as succinate occurs via an electrogenic process. Exposure of NaCT-expressing oocytes to citrate or succinate is associated with inward currents in the presence of Na^+ , indicating that, for citrate as well as succinate, the transport process results in the transfer of net positive charge into the oocytes. But, the amount of positive charge transferred into the oocytes varies depending on whether the transported substrate is citrate or succinate. With citrate, the charge-to-substrate ratio is 1, indicating that citrate transport is associated with the transfer of one positive charge per citrate molecule. Therefore, the Na^+ :citrate stoichiometry should be 3:1 if citrate is recognized by the transporter as a divalent anion or 4:1 if the trivalent anionic form is recognized instead.

In contrast to citrate, the charge-to-substrate ratio is 2 for succinate that indicates that succinate transport is associated with the transfer of two positive charges per succinate molecule. Since succinate exists predominantly as a divalent anion under the experimental conditions, the Na^+ :succinate stoichiometry should be 4:1. Taken collectively, the data indicate that mouse NaCT transports succinate as well as citrate by an electrogenic process with a Na^+ :substrate stoichiometry of 4:1. Furthermore, the charge-to-substrate ratio of 2 for succinate and 1 for citrate strongly suggests that NaCT is capable of transporting citrate as a trivalent anion and succinate as a divalent anion. Thus, NaCT is a Na^+ -coupled transporter for dicarboxylates as well as tricarboxylates. This characteristic is distinct from that of NaDC1 and NaDC3 which recognize their substrates only in their divalent anionic form.

TABLE 9
Exon-intron organization of mouse *nact* gene

5' Exon			Intron			3' Exon		
No.	Size (bp)	Sequence	Donor	Size (bp)	No.	Acceptor	Sequence	No.
1	>118	...GACAAG	gtcagt...	4312	1	...cttcag	TTTGCC...	2
2	129	...AAGCAG	gtgagt...	273	2	...ctccag	GTATGT...	3
3	137	...CTCACG	gtaaac...	1230	3	...ctgcag	GCTGAT...	4
4	188	...TGCCAG	gtgcac...	1068	4	...ccacag	GAAGCC...	5
5	169	...GCAGGA	gtgagt...	1475	5	...tttcag	ATTGTT...	6
6	123	...ACACAA	gtaagt...	3957	6	...gtctag	TTTAAA...	7
7	219	...CACCGT	gtaagt...	825	7	...tatcag	TCATAT...	8
8	101	...AGGAAG	gtaagg...	1413	8	...tcgcag	AAAGGA...	9
9	119	...TGTGAG	gtactt...	2897	9	...tcctag	ACGTCA...	10
10	162	...TCCATG	gtaagt...	2403	10	...ctctag	GCTCGT...	11
11	138	...GACATG	gtaaca...	1489	11	...ccctag	ATGAAA...	12
12	>132	...ACTTAG...						

TABLE 10
Substrate specificity of mouse NaCT

Substrate analog	[¹⁴ C]-Citrate transport		[³ H]-Succinate transport	
	pmol/10 ⁶ cells/min	%	pmol/10 ⁶ cells/min	%
Control	38.4 ± 6.0	100	0.145 ± 0.010	100
Citrate	0.7 ± 0.1	2	0.004 ± 0.001	3
Succinate	0.1 ± 0.1	1	0.007 ± 0.003	5
Fumarate	1.6 ± 0.4	4	0.007 ± 0.002	5
Malate	0.2 ± 0.1	1	0.004 ± 0.001	3
2-Oxo-glutarate	4.6 ± 0.5	12	0.018 ± 0.001	12
Maleate	31.2 ± 2.5	82	0.165 ± 0.014	113
Isocitrate	26.3 ± 1.1	69	0.125 ± 0.003	86
<i>Cis</i> -Aconitate	18.1 ± 1.8	47	0.062 ± 0.005	43
Pyruvate	32.3 ± 1.5	84	0.151 ± 0.013	104
Lactate	29.8 ± 3.4	78	0.162 ± 0.008	111

- 5 Transport of [¹⁴C]-citrate (10 μM) and [³H]-succinate (0.1 μM) was measured in parallel in vector-transfected cells and in cells transfected with mouse NaCT cDNA in the absence or presence of 2 mM various substrate analogs. NaCT-specific transport was calculated by subtracting the transport in vector-transfected cells from the transport in cDNA-transfected cells. Data (mean ±
- 10 S.E.) represent only the NaCT-specific transport.

The electrophysiological data from the oocyte expression studies demonstrate that the number of Na⁺ involved in the transport process is 4 irrespective of whether the transported substrate is citrate or succinate. In

15 contrast, the Na⁺-activation kinetics in mammalian cell expression studies indicated that the number of Na⁺ ions involved in the transport process is 3 if the transported substrate is citrate and that the number changes to 2 if the transported substrate is succinate. However, since the oocyte expression studies demonstrate unequivocally the eletrogenic nature of the transport process for

succinate which exists almost entirely as a divalent anion under the experimental conditions, the Na^+ :succinate stoichiometry of 2 cannot be correct.

Obviously, the value obtained for the number of Na^+ ions involved in the transport of succinate in the mammalian cell expression system is an underestimate. The value of 3 for the Na^+ :citrate stoichiometry can be reconciled with the electrogenic nature of the transport process if one hypothesizes that citrate is transported as a divalent anion. But, the charge-to-substrate transfer ratios calculated from the oocyte expression studies suggest that citrate is recognized as a trivalent anion while succinate is recognized as a divalent anion. Therefore, the value obtained for the number of Na^+ ions involved in the transport of citrate in the mammalian cell expression system is also likely to be an underestimate. There is precedence for such discrepancies between the Na^+ :substrate coupling ratio determined from Na^+ -activation kinetics (i.e., the Hill coefficient) and the actual coupling ratio (Pajor and Sun, *Am. J. Physiol.* (2000);279: F482-F490 and Pajor et al., *Am. J. Physiol.* (2001);280: C1215-C1223). For mouse NaDC1 and NaDC3, the Hill coefficient calculated from Na^+ -activation kinetics is 2 with succinate as the substrate while the transport process is undoubtedly electrogenic. The electrogenicity of the transport process mandates that the Na^+ -to-succinate coupling ratio should be at least 3. This apparent discrepancy arises because the Hill coefficient does not always represent the absolute value. The Hill coefficient represents the minimal number of Na^+ binding sites involved in the activation process and the value for the Hill coefficient determined from Na^+ -activation kinetics is influenced significantly by the strength of cooperativity among the binding sites.

Another significant difference observed in the mammalian cell expression system is in the nature of Na^+ -activation kinetics between the transport of succinate and citrate. The transport of citrate saturates within the concentration range of Na^+ tested whereas the transport of succinate does not saturate under identical conditions. Apparently, the cooperativity among the different Na^+ -binding sites differs depending on whether the transported substrate is succinate or citrate. Whether this is due to the difference in the

ionic nature (i.e., divalent nature of succinate and the trivalent nature of citrate) or the bulkiness of these two substrates is not known.

The substrate specificity of mammalian NaCTs merits discussion. Human NaCT appears to be rather specific for citrate as its interaction with succinate and other dicarboxylates occurs with a very low affinity. In contrast, both rat and mouse NaCTs interact with citrate as well as succinate with comparable affinity. In this respect, the rodent NaCTs are similar to *Drosophila* NaCT (Indy). This functional difference can be used as a diagnostic criterion for the identification of the protein domains involved in substrate binding using human-mouse or human-rat chimeric NaCTs. A similar approach has been used to obtain valuable information on the substrate binding site of other transporters such as NaDC3 (Wang et al., *Am. J. Physiol.* (2000);278: C1019-C1030) and OCTN2 (Seth et al., *J. Biol. Chem.* (1999);274: 33388-33392).

Example 6

Zebrafish Na⁺-coupled Citrate Transporter (NaCT):

Primary Structure and Transport Function

The zebrafish Na⁺-coupled citrate transporter (NaCT) has been cloned and functionally characterized. The nucleotide sequence of the full-length cDNA clone and translated amino acid sequence of the zebrafish Na⁺-coupled citrate transporter are SEQ ID NO:11 and SEQ ID NO:12, respectively, are shown in Fig. 36.

A comparison of the amino acid sequence of zebrafish NaCT (SEQ ID NO:12) with that of rat (SEQ ID NO:4), mouse (SEQ ID NO:10), and human (SEQ ID NO:6), carried out as described in Examples 1-5, is shown in Fig. 37. Zebrafish NaCT is 61% identical and 77% similar to human NaCT. Zebrafish NaCT is 57% identical and 72% similar to rat NaCT. Zebrafish NaCT is 57% identical and 74% similar to mouse NaCT.

Using the methods described in Examples 1-5, the zebrafish clone was determined to be fully functional. Fig. 38A shows a time course of citrate (2

μM) uptake in cells transfected with either vector alone or zebrafish NaCT cDNA and Fig. 38B demonstrates the influence of pH on citrate (1 μM) uptake mediated by zebrafish NaCT. Saturation kinetics (Fig. 39A) and Na⁺-activation kinetics (Fig. 39B) of citrate uptake mediated by zebrafish NaCT. The Michaelis constant for citrate uptake is 40 ± 4 μM. The value for Hill coefficient for the activation of uptake is 26 ± 0.2. Fig. 40A shows the inhibition of zebrafish NaCT-mediated [¹⁴C]-citrate (1 μM) uptake by various structural analogs (2mM). Fig. 40B demonstrates dose-response relationships for inhibition of zebrafish NaCT-mediated [¹⁴C]-citrate (1 μM) uptake by citrate, succinate, and cis-aconitate. The IC₅₀ values for the inhibition (i.e., the concentration of the inhibitor necessary for 50% inhibition) are 30 ± 4, 51 ± 9, and 624 ± 45 μM, respectively, for citrate, succinate, and cis-aconite.

15

Example 7

Human NaCT, the Ortholog of *Drosophila* Indy, as a Novel Target for Lithium Action

NaCT is a Na⁺-coupled citrate transporter recently identified in mammals that mediates the cellular uptake of citrate. It is expressed predominantly in the liver. NaCT is structurally and functionally related to the product of the *indy* gene in *Drosophila* whose dysfunction leads to lifespan extension. This example shows that NaCT mediates the utilization of extracellular citrate for fat synthesis in human liver cells and that the process is stimulated by lithium. The transport function of NaCT is enhanced by lithium at concentrations found in humans treated with lithium for bipolar disorder. Valproate and carbamazepine, two other drugs that are used for the treatment of bipolar disorder, do not affect the function of NaCT. The stimulatory effect of Li⁺ is specific for human NaCT as NaCTs from other animal species are either inhibited or unaffected by Li⁺. The data suggest that two of the four Na⁺-binding sites in human NaCT may become occupied by Li⁺ to produce the stimulatory effect. The stimulation of NaCT in humans by lithium at therapeutically relevant concentrations has potential clinical implications. It is

also show here that a single base mutation in codon-500 (TTT→CTT) in human NaCT, leading to the substitution of Phe with Leu, stimulates the transport function and abolishes the stimulatory effect of lithium. This raises the possibility that genetic mutations in humans may lead to alterations in the constitutive activity of the transporter with associated clinical consequences.

As shown in Example 1, the *Indy* gene in *Drosophila* codes for a plasma membrane transporter that transports various dicarboxylates such as succinate as well as the tricarboxylate citrate. Dysfunction of this gene leads to lifespan extension in this organism (Rogina et al., *Science* (2000);290: 2137-2140). The search for the mammalian ortholog of *Drosophila* Indy has identified NaCT in rat (Example 2) and human (Example 3) tissues as the plasma membrane transporter with functional characteristics similar to those of Indy. NaCT belongs to the sodium dicarboxylate/sulfate cotransporter gene family (*SLC13*) (Pajor, *J. Membr. Biol.* (2000);175: 1-8). While the previously identified mammalian sodium dicarboxylate cotransporters NaDC1 and NaDC3 recognize only dicarboxylates as substrates, the newly identified NaCT accepts various dicarboxylates as well as the tricarboxylate citrate as substrates. In fact, human NaCT transports citrate much more effectively than succinate. The Na⁺:substrate stoichiometry for NaCT is 4:1 irrespective of whether the transported substrate is a dicarboxylate or a tricarboxylate. In contrast, the Na⁺:substrate stoichiometry for NaDC1 and NaDC3 is 3:1 (Pajor, *J. Membr. Biol.* (2000);175: 1-8). Earlier studies have shown that NaDC1 and NaDC3 are inhibited by lithium (Pajor, *J. Membr. Biol.* (2000);175: 1-8 and Wang et al., *Am. J. Physiol.* (2000);278: C1019-C1030). Urinary excretion of dicarboxylates is increased significantly in patients undergoing lithium therapy for affective disorders (Bond et al., *Br. J. Pharmacol.* (1972);46: 116-123) and inhibition of NaDC1 in the kidney by Li⁺ is believed to be the cause for this phenomenon (Wright et al., *Proc. Natl. Acad. Sci.* (1982);79: 7514-7517).

Since NaCT is structurally and functionally similar to NaDCs, this example investigated whether the transport function of NaCT is also inhibited by lithium. These studies have led to unexpected findings. While NaCTs from non-human organisms are either inhibited or unaffected by Li⁺, human NaCT is markedly stimulated by Li⁺. This example identifies human NaCT as a novel

target for lithium action and have potential clinical implications for individuals who are on lithium therapy for affective disorders and also shows that NaCT facilitates the utilization of extracellular citrate for lipid synthesis in human liver cells and that Li^+ stimulates this process. Furthermore, using rat/human chimeric NaCTs and site-directed mutagenesis, it is demonstrated here that a single base mutation in codon-500 (TTT→CTT) in human NaCT, leading to the substitution of Phe with Leu, stimulates the transport function and abolishes the stimulatory effect of lithium.

10 MATERIALS AND METHODS

Heterologous expression of NaCTs. NaCT cDNAs cloned from human hepatocarcinoma cell line HepG2 (SEQ ID NO:5), rat brain (SEQ ID NO:3), mouse liver (SEQ ID NO:9), whole zebra fish (SEQ ID NO:11), and whole *C. elegans* (SEQ ID NO:7) were expressed functionally in a human retinal pigment epithelial cell line (HRPE) using the vaccinia virus expression technique as described previously in Examples 3 and 5.

The same approach was also used to analyze the function of chimeric and mutant NaCTs. The transport function of NaCTs was monitored by measuring the uptake of [^{14}C]citrate (Moravek Biochemicals, Brea, CA) using the uptake medium containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 5 mM glucose, buffered with 25 mM Hepes/Tris, pH 7.5. Uptake measurements were made in parallel in cDNA-transfected cells and in cells transfected with vector alone. The uptake in vector-transfected cells (endogenous activity) was subtracted from the uptake in cDNA-transfected cells to determine the cDNA-specific activity. When the effect of Li^+ was studied, the uptake medium contained either LiCl or equimolar concentrations of *N*-methyl-D-glucamine chloride. The Na^+ activation kinetics were analyzed by measuring the uptake of citrate at increasing concentrations of Na^+ , with the concentration of *N*-methyl-D-glucamine chloride adjusted appropriately to maintain the osmolality of the uptake medium. The Hill coefficient (n_{H} ; the number of Na^+ ions involved in the activation process) was determined by fitting the data to Hill equation. A 30-minute incubation was used in uptake

measurements as these experimental conditions have been shown to be suitable for measurement of initial uptake rates for NaCTs.

Uptake measurement in human liver cell lines. The human liver cell lines (HepG2 and Huh-7) were cultured to confluency in 24-well culture plates using appropriate culture medium. The HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA). The Huh-7 cell line (Bode et al., (2002) *Am. J. Physiol.* 283, G1062-G1073) was provided by Dr. Barrie P. Bode (St. Louis University, St. Louis, MO). Uptake measurements in monolayer cultures of these cells were measured as described above for the heterologously expressed NaCTs.

Measurement of incorporation of citrate and acetate into cellular lipids. Monolayer cultures of HepG2 cells were incubated with [^{14}C]citrate (0.1 μCi) or [^{14}C]acetate (0.1 μCi) (American Radiolabeled Chemicals, St. Louis, MO) for 24 hours in a NaCl-containing medium in the presence or absence of 2 or 10 mM Li^+ . The cellular lipids were then extracted by n-hexane/isopropanol (3:2, v/v) as described by Scharnagl et al. (Scharnagl et al., (2001) *Biochem. Pharmacol.* 62, 1545-1555). The radioactivity associated with the lipid fraction was quantified to calculate the extent of incorporation of extracellularly added [^{14}C]citrate or [^{14}C]acetate into lipids.

RESULTS AND DISCUSSION

First, the influence of Li^+ on the transport function of rat NaCT was tested. These studies showed that the activity was indeed inhibited by Li^+ (Fig. 41). However, when the effect of Li^+ on the activity of human NaCT was tested, the results were quite unexpected. The transport activity of human NaCT was not inhibited but stimulated by Li^+ (Fig. 41). Li^+ caused marked inhibition of citrate uptake via rat NaCT with an IC_{50} of 2.1 ± 0.8 mM. In contrast, Li^+ stimulated citrate uptake via human NaCT and the concentration of Li^+ needed for half-maximal stimulation was 2.1 ± 0.7 mM. The plasma concentrations of Li^+ in patients treated with lithium are in the range of 0.8–2 mM (Sproule, *Clin. Pharmacokinet.* (2002);41: 639-660). At a concentration of 2.5 mM, Li^+ stimulated human NaCT activity by 2.4-fold. The stimulatory effect of Li^+ is associated with an increase in substrate affinity as well as with a

decrease in maximal velocity (Fig. 42A). In the presence of 10 mM Li^+ , the affinity for citrate increased approximately 7-fold (K_t in the absence of Li^+ , $702 \pm 55 \mu\text{M}$; K_t in the presence of Li^+ , $95 \pm 15 \mu\text{M}$). The normal concentrations of citrate in human blood are in the range of 100–150 μM (Nordmann and
5 Nordmann, *Adv. Clin. Chem.* (1961);4: 53-120). Therefore, under physiological conditions, the increase in substrate affinity is the primary effect of Li^+ on cellular entry of citrate via NaCT. Li^+ cannot support NaCT function in the absence of Na^+ . The stimulation of human NaCT function by Li^+ was observed only in the presence of Na^+ . The stimulatory effect was greater at lower
10 concentrations of Na^+ and the effect decreased as the concentration of Na^+ increased (Fig. 42B). The stimulation was 3-fold at 140 mM Na^+ , but the stimulation was 7-fold in the presence of 60 mM Na^+ . The Na^+ :citrate stoichiometry was also analyzed for the transport process in the presence and absence of Li^+ by fitting the data from the Na^+ -activation kinetics to Hill
15 equation. The Hill coefficient (n_H), which represents the number of Na^+ ions involved in the activation process, was 4.5 ± 0.7 in the absence of Li^+ . This value changed to 1.8 ± 0.1 in the presence of 10 mM Li^+ . Measurements of citrate transfer and charge transfer in *X. laevis* oocytes expressing mammalian NaCT have shown that the Na^+ :citrate stoichiometry for NaCT is 4:1 and the
20 Hill coefficient of 4.5 ± 0.7 determined in the present study is close to the actual value determined in oocytes. Interestingly, the Na^+ :citrate stoichiometry changes to 2:1 in the presence of Li^+ . These data suggest that two of the four Na^+ -binding sites in human NaCT may become occupied by Li^+ during the stimulatory process.

25 These data show that there is a species-specific influence of Li^+ on the activity of NaCT. To analyze this species-specific phenomenon in a broader scope, the NaCT orthologs from three additional species: mouse, zebra fish, and *C. elegans* were cloned. NaCTs from these species also mediate Na^+ -coupled transport of citrate. The influence of Li^+ on the function of these NaCTs (Table
30 11) was then tested. The transport function of mouse and zebra fish NaCTs were inhibited by Li^+ as was rat NaCT. In contrast, NaCT from *C. elegans* was not affected by Li^+ . To confirm the stimulatory effect of Li^+ on human NaCT seen with the cloned transporter, the effect of Li^+ on constitutively expressed

NaCT in human liver cells was studied. For this purpose, two different human hepatoma cell lines (HepG2, and Huh-7) were used. In both cell lines, the constitutive expression of NaCT was detectable as measured by Na⁺-coupled uptake of citrate. Li⁺ stimulated this citrate uptake activity in both cell lines
5 (Table 11).

In addition to lithium, two other drugs, valproate and carbamazepine, are currently used for effective treatment of bipolar disorder. The activity of human NaCT was not affected by therapeutically relevant concentrations of these two drugs at (valproate, 0.6 mM; carbamazepine, 50 μM) (Table 11). The
10 therapeutic plasma levels for valproate and carbamazepine in humans are in the range of 0.3-0.6 mM and 20-50 μM, respectively (Williams et al. (2002) *Nature* 417, 292-295). Therefore, the concentrations of valproate and carbamazepine used in the present study are clinically relevant. The lack of effect of valproate and carbamazepine on human NaCT was also evident with the constitutively
15 expressed NaCT in HepG2 cells. These drugs had no effect on not only human NaCT but also on NaCTs cloned from other species (Table 11). Thus, among the three widely used mood stabilizers, only lithium has the stimulatory effect on human NaCT.

NaCT is the first plasma membrane transporter identified in mammals
20 that shows preference for citrate as a substrate and operates very effectively under physiological concentrations of citrate in the circulation. Citrate has wide biological functions. It is not only an intermediate in the citric acid cycle and hence a critical substrate for energy production, but also is a source of acetyl CoA in the cytoplasm for the synthesis of cholesterol and fatty acids. Citrate
25 levels in the cytoplasm also signal the energy status of the cell by serving as a potent inhibitor of phosphofructokinase-1, the rate-limiting enzyme in glycolysis. Since the concentrations of citrate in blood are quite high, the presence of a plasma membrane transporter for citrate will provide the cells a continuous supply of an additional source of citrate for utilization in metabolic
30 pathways. Therefore, the recent identification of NaCT as a plasma membrane energy-coupled uphill transporter for citrate in mammals is of great biological relevance. The functional characteristics and the tissue distribution pattern of NaCT support a critical role for this transporter in cellular metabolism. The

affinity of human NaCT for citrate is ideal for its role in mediating the cellular entry of citrate from blood (K_t value, approximately 700 μM ; citrate levels in human blood, approximately 135 μM). The transporter is expressed predominantly in the liver. It is also expressed in the testis and brain, but at much lower levels. Liver is metabolically very active, especially in the synthesis of cholesterol and fatty acids and in glucose homeostasis, the biochemical pathways in which citrate plays a critical role. *In situ* hybridization studies show that NaCT mRNA is expressed uniformly in perivenous hepatocytes as well as in periportal hepatocytes. These findings are of interest, considering the metabolic role of citrate in fatty acid synthesis, cholesterol synthesis, glycolysis, and gluconeogenesis. Citrate is not only a precursor for the synthesis of fatty acids and cholesterol but also a potent inhibitor of phosphofructokinase-1, one of the rate limiting enzymes in the glycolytic pathway. Therefore, citrate is an inhibitor of glycolysis and stimulator of gluconeogenesis. These four metabolic processes, namely fatty acid synthesis, cholesterol synthesis, glycolysis, and gluconeogenesis, exhibit zonal heterogeneity in the liver. Fatty acid synthesis and glycolysis occur predominantly in the perivenous hepatocytes whereas cholesterol synthesis and gluconeogenesis occur predominantly in the periportal hepatocytes (Jungermann and Katz, *Physiol. Rev.* (1989);69: 708-764). The observations that NaCT is expressed in perivenous as well as periportal hepatocytes suggest a role for this transporter in all of these metabolic processes irrespective of the zone-specific occurrence of these processes in the liver.

If NaCT plays a role in facilitating the hepatic utilization of extracellular citrate for the synthesis of fatty acids and cholesterol, the incorporation of extracellular citrate into lipids would be enhanced by Li^+ in human liver cells. To test this, the incorporation of [^{14}C]citrate from extracellular medium into lipid fraction that is extractable with n-hexane/isopropanol in HepG2 cells was monitored and the influence of Li^+ on the process was assessed. The extracted lipid fraction contains triglycerides, cholesterol esters, and cholesterol. These studies have shown that Li^+ markedly enhances the incorporation of citrate into this lipid fraction (Fig. 43). The stimulation was 2.6 ± 0.1 -fold at therapeutically relevant concentrations of Li^+ (2 mM). There are additional

targets for Li^+ in mammalian cells. Inositol monophosphatase, inositol
 polyphosphatase, and glycogen synthase kinase-3 β are considered to be the
 primary targets for lithium action related to the therapeutic efficacy of lithium in
 the treatment of bipolar disorder (Williams et al., *Nature* (2002);417: 292-295;
 5 Williams and Harwood, *Trends Pharmacol. Sci.* (2000);21: 61-64; Phiel and
 Klein, *Annu. Rev. Pharmacol. Toxicol.* (2001);41: 789-813; and Li et al.,
Bipolar Disord. (2002);4: 137-144). Of these, glycogen synthase kinase-3 β is
 involved in hepatic energy metabolism. Therefore, it is important to establish
 that the observed Li^+ -induced increase in the incorporation of extracellular
 10 citrate into lipids does not occur through targets other than NaCT. To provide
 evidence for the involvement of NaCT in this process, the incorporation of
 extracellular [^{14}C]acetate into lipids in HepG2 cells was monitored and the
 influence of Li^+ on this process was assessed (Fig. 43). NaCT has no role in the
 entry of acetate into hepatocytes. Extracellular acetate was incorporated into
 15 lipids extractable with n-hexane/isopropanol as expected, but Li^+ did not have
 any effect on this process. These data show that Li^+ at therapeutic
 concentrations enhances the utilization of extracellular citrate in human liver
 cells for the synthesis of fatty acids and cholesterol specifically via stimulation
 of NaCT.

20 To determine the domains in human NaCT that are responsible for the
 stimulation of transport function by Li^+ , 28 different chimeric transporters
 consisting of different regions of human NaCT and rat NaCT were made and
 compared their transport function with that of the parent rat and human NaCTs.
 The results of these studies have shown that replacement of a small region
 25 (amino acid position 496-516) in human NaCT with the corresponding region
 (amino acid position 500-520) in rat NaCT stimulated the transport function 5-
 fold and activity of this chimeric transporter is not affected by Li^+ (Fig. 44A).
 There are only two amino acid differences between human and rat NaCTs in
 this region (Phe versus Leu and Thr versus Ala) (Fig. 44B). Codon-500 and
 30 codon-516 were individually mutated in human NaCT to generate the Phe \rightarrow Leu
 and Thr \rightarrow Ala mutants that match the sequence in rat NaCT in this region. The
 Thr \rightarrow Ala mutation did not affect the transport activity nor did it interfere with
 the Li^+ -dependent stimulation. In contrast, the Phe \rightarrow Leu mutation

(TTT→CTT) led to a 3- to 4-fold stimulation of transport activity. Kinetic analysis of the wild type human NaCT and its Phe→Leu mutant showed that the stimulation of transport function seen with the mutant was due to an increase in substrate affinity with very little change in maximal velocity (Fig. 44C). The wild type human NaCT showed a K_t value of $585 \pm 87 \mu\text{M}$ for citrate. The corresponding value for the mutant was $78 \pm 8 \mu\text{M}$. Thus, the Phe→Leu mutant showed a 7-fold increase in substrate affinity. Since a single base pair change in the codon can bring about this amino acid substitution (TTT→CTT), these findings suggest that genetic mutations in humans have potential to effect marked changes in the constitutive activity of NaCT and that such changes may influence the role of the transporter in hepatic synthesis of triglycerides and cholesterol. Interestingly, the Phe→Leu mutant did not exhibit Li^+ sensitivity (Fig. 44D). The transport function of wild type human NaCT was stimulated by Li^+ whereas the transport function of the mutant, which was about 4-fold higher than that of wild type NaCT, was not affected by Li^+ . Since rat NaCT, which possesses Leu at this site, is inhibited by Li^+ , the findings that activity of the Phe→Leu mutant of human NaCT is not inhibited by Li^+ indicate that this region may not represent the Li^+ -binding site. Furthermore, Li^+ affects the transport function of human NaCT not only by increasing the substrate affinity but also by decreasing the maximal velocity (Fig. 42A). In contrast, the influence of the Phe→Leu mutation is solely on substrate affinity. While the domain in human NaCT consisting of the amino acids 496-516 definitely plays a critical role in substrate binding, it may not have a direct role in Li^+ binding. But, this domain appears to interact with the Li^+ -binding site, thus modifying the influence of Li^+ on the transport function. Multiple alignments of amino acid sequences of NaCTs from different species using the program "T-coffee," available on the world-wide web at ch.embnet.org/software/TCoffee.html, show that the amino acid corresponding to Phe-500 in human NaCT is Leu in rat and mouse NaCTs. Interestingly, the corresponding amino acid in zebra fish and *C. elegans* NaCTs is Phe. Nonetheless, zebra fish NaCT was inhibited by Li^+ , whereas the *C. elegans* NaCT was not influenced by Li^+ . This agrees with the conclusion that the domain containing this amino acid does not represent the Li^+ -binding site.

This example shows that Li^+ is a potent activator of NaCT, at concentrations found in patients treated with lithium for affective disorders, have important clinical implications. Interestingly, the activation of NaCT by Li^+ is seen only with human NaCT. Patients with bipolar disorder are treated effectively with either lithium or other mood stabilizers such as valproate and carbamazepine. Present studies show that the activation of human NaCT is seen only with lithium and not with valproate and carbamazepine. Thus, only those patients who take lithium face the clinical consequences of activation of NaCT function. Such consequences include the enhanced synthesis of cholesterol and fatty acids and consequently an increased risk for hypercholesterolemia, hyperlipidemia, hyperglycemia, obesity, and insulin-resistant diabetes. It has been known for some time that lithium therapy is associated with significant increase in body weight (Chen and Silverstone, *Int. Clin. Psychopharmacol.* (1990);5: 217-225; Coxhead et al., *Acta Psychiatr. Scand.* (1992);85: 114-118; Price and Heninger, *N. Engl. J. Med.* (1994);331: 591-598; Baptista et al., *Pharmacopsychiatry* (1995);28: 35-44; Fagiolini et al., *J. Clin. Psychiatry* (2002);63: 528-533; and Atmaca et al., *Neuropsychobiology* (2002);46: 67-69).

Inositol monophosphatase, inositol polyphosphatase, and glycogen synthase kinase-3 β , the previously known targets for Li^+ , are all enzymes and are all inhibited by Li^+ . Human NaCT is, in contrast, a plasma membrane transporter that is activated rather than inhibited by Li^+ . Human NaCT thus represents a new and novel target for Li^+ .

The data with the Phe \rightarrow Leu mutant of human NaCT raise the possibility that genetic mutations in this amino acid position may alter the constitutive activity of the transporter in humans and consequently influence the hepatic utilization of extracellular citrate for fatty acid and cholesterol synthesis. Individuals with such mutations are likely to exhibit significant alterations in their blood lipid profile.

TABLE 11
Differential effects of Li⁺, valproate, and carbamazepine on NaCT from
different species and on NaCT in human liver cell lines.

	Control	Li ⁺ (2 mM)	Valproate (0.6 μM)	Carbamazepine (50 μM)
<i>Citrate uptake (pmol/10⁶ cells/min)</i>				
hNaCT	51.8 ± 2.5 (100)	105.2 ± 11.6 (203)	57.3 ± 2.6 (111)	54.1 ± 2.6 (105)
rNaCT	148.8 ± 7.2 (100)	92.3 ± 8.4 (62)	142.4 ± 6.3 (96)	133.5 ± 0.7 (90)
mNaCT	42.5 ± 2.3 (100)	29.4 ± 1.7 (69)	42.5 ± 4.4 (100)	43.7 ± 2.0 (103)
ceNaCT	43.5 ± 2.1 (100)	43.6 ± 0.8 (100)	46.0 ± 3.6 (106)	45.1 ± 1.5 (104)
zfNaCT	485.8 ± 20.6 (100)	298.0 ± 12.6 (61)	453.0 ± 26.6 (93.2)	441.0 ± 2.7 (91)
<i>Citrate uptake (pmol/min/mg protein)</i>				
HepG2 cells	9.5 ± 0.3 (100)	21.2 ± 0.6 (222)	9.9 ± 0.3 (105)	10.0 ± 0.2 (106)
Huh-7 cells	10.2 ± 0.2 (100)	20.0 ± 0.5 (196)	ND	ND

5

Values in parentheses represent percent of corresponding control. ND, not determined.

Example 8
Inhibition of Citrate Transport via the NaCT Citrate Transporter by
Hydroxycitrate

5 Human and rat NaCT cDNAs were independently expressed in HRPE cells and the transport function of the expressed transporter was assessed by the measurement of citrate into cells using the methods described in Example 2 and Example 3. The inhibitory potency of hydroxycitrate was monitored by determining citrate uptake into the cells in the absence (control) and presence of
10 0.1 mM hydroxycitrate. At this concentration, hydroxycitrate inhibited citrate uptake into the cells via NaCT by about 30-40%. These data show that hydroxycitrate is an inhibitor of the citrate transporter.

 The complete disclosure of all patents, patent applications, and
15 publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given
20 for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

 All headings are for the convenience of the reader and should not be
25 used to limit the meaning of the text that follows the heading, unless so specified.

Sequence Listing Free Text

	SEQ ID NO:1	cDNA of Drosophila drINDY
	SEQ ID NO:2	amino acid sequence of Drosophila drINDY
5	SEQ ID NO:3	cDNA of rat NaCT
	SEQ ID NO:4	amino acid sequence of rat NaCT
	SEQ ID NO:5	cDNA human NaCT
	SEQ ID NO:6	amino acid sequence of human NaCT
	SEQ ID NO:7	cDNA of C. elegans NaCT
10	SEQ ID NO:8	amino acid sequence of C. elegans NaCT
	SEQ ID NO:9	cDNA of mouse NaCT
	SEQ ID NO:10	amino acid sequence of mouse NaCT
	SEQ ID NO:11	cDNA of zebrafish NaCT
	SEQ ID NO:12	amino acid sequence of zebrafish NaCT
15	SEQ ID NO:13	amino acid sequence of rat NaDC1
	SEQ ID NO:14	amino acid sequence of rat NaDC3
	SEQ ID NO:15-28	artificially synthesized oligonucleotide primers
	SEQ ID NO:29	peptide consensus sequence